COMBINATION METHODS FOR MICROBIAL DECONTAMINATION

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This is the original works of the author

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In the name of God

This thesis is dedicated to the memory of my father

A Prayer

With uplifted arms I pray
God all knowing, show me Thy way
Make me a channel of Thy light
Bring to this earth much delight
And help me strive to love like Thee
And through wisdom, other gods flee.
Break the illusion, clearly see
And try to live my destiny.
Oh God help me know forever Thee
Are close to me, with me, in me.

Shahriar Shahriari

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ABSTRACT

Novel methods to improve the microbiological quality and to extend the shelf life of foods would be advantageous. In this study, the killing effect of UV, laser, microwave radiation, conventional heating and ozone was investigated, alone and in various combinations, on saline suspensions and agar plate cultures of *Listeria monocytogenes, Salmonella typhimurium, Campylobacter jejuni, Shewanella putrefaciens, Pseudomonas fragi, Micrococcus luteus* and on *E. coli (lux)* as an indicator organism. *E. coli (lux)* was the most sensitive to the effect of UV, whereas *M. luteus* was the most resistant to UV and Nd:YAG laser radiation. *S. putrefaciens* was the most sensitive bacterium to Nd:YAG laser radiation. With microwave treatment, a temperature between 70-71°C was the critical point for killing bacteria by microwave energy, although there was evidence of an athermal effect of microwave on bacteria. Ozone was effective against the bacteria used, although the killing of bacteria on the foodstuffs was less significant than killing on plates.

The killing effect of Nd:YAG laser and CO_2 laser was also investigated on different bacteria on agar plates. Higher frequencies of the Nd:YAG laser resulted in improved clearing effects and, with the CO_2 laser, continuous wave always showed better clearing compared to pulsed wave. In comparison of the two laser types, the energy density needed for the Nd:YAG laser was approximately 300 times more than that needed by the CO_2 laser to produce the same clear area on the agar plates.

Sequential treatment of bacterial suspensions by UV, microwave/conventional heating and Nd:YAG laser gave much greater killing than the sum of the effect of the three treatments alone. Also, greater killing was apparent with the order: laser, microwave/conventional heating and UV compared to the order: microwave/conventional heating, UV and laser. Under standard conditions, the priority of the order L+H+UV over the order H+UV+L was seen consistently through the different experiments and the difference was statistically significant. Differences between the best and worst orders of treatment were increased when more severe treatment conditions were used. Results showed that killing by the sequential treatments on bacteria applied to smoked salmon was almost the same as that for the sum of the three treatments alone.

The bioluminescent bacterial strain, *E. coli (lux),* was investigated as an indicator organism as part of a possible real-time method of measuring the efficiency of the different treatments and combinations. UV reduced the viability of the bacterium by about 8 logs, but the light output immediately after treatment was not significantly affected by UV treatment. In contrast, laser treatment and conventional heating reduced the light output dramatically without greatly reducing the subsequent viable count. These observations highlight limitations of the use of the bioluminescence technique as a real-time monitor of bacterial viable cell numbers. However, under standard conditions, perhaps with a more highly bioluminescent organism, it is possible that the method could be useful in the study of particular decontamination processes.

In this study, the killing mechanisms by different treatments were investigated. It was shown that cell constituents released by one method of treatment could protect bacteria against subsequent treatments. In another investigation, release of nucleic acid and protein by different treatments varied and in general, the greater the killing effect produced, the greater the release of material. Only killing by UV did not release a significant amount of nucleic acid and protein. These results suggest that each treatment caused different types of damage and has a different killing mechanism.

A quick freezing of the bacterial suspensions after microwave treatment increased their susceptibility to the killing effect of subsequent treatment(s). This method could be suggested as a part of a decontamination procedure in the food processing industry but needs more investigation.

Laser, microwave or conventional heating sensitise the bacteria to lysis by SDS, but these effects were lower for ozone and minimal for UV treatment. *L. monocytogenes* was highly sensitive to SDS and also there was a synergistic effect between SDS and other treatments on killing of the bacterium. So, SDS or similar detergents could be used in decontamination of seafood factories or other materials and surfaces.

By scanning and transmission electron microscopy, no gross ultrastructural changes to the internal structures of the cell or rupture of the cell-envelope of *E. coli* were observed with the different treatments.

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LIST OF ABBREVIATIONS

cfu	colony forming units
CO_2	carbon dioxide
ED	energy density
F	frequency
FDA	US Food and Drug Administration
g	grams
h	hours
Hz	Hertz
J	Joules
kW	kilowatts
mg	milligram
MHz	mega Hertz
ml	millilitre
min	minutes
mW	milliwatts
µl	microlitre
µW	microwatts
NaCl	sodium chloride
Nd:YAG	neodymium:yttrium aluminium garnet
NPN	none-protein nitrogen
O_3	ozone
O_2	oxygen
OD	optical density
PRF	pulse repetition frequency
rpm	revolutions per minute
sec	seconds
SDS	sodium dodecyle sulphate
SEM	scanning electron microscopy
STDEV	standard deviation
TEM	transmission electron microscopy
TMA	trimethylamine

TMAO	trimethylamine oxide
USDA	US Department of Agriculture
UV	ultraviolet
W	Watts

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Chapter 1

INTRODUCTION

CHAPTER 1 INTRODUCTION

1.1 Background

While the natural resources available for food production are shrinking, the world's population goes on increasing. Unfortunately every year tonnes of food are spoiled by physical, chemical and microbial activities. However, by far the majority of losses results from the effect of microorganisms. Microbial food spoilage ends in the loss of its original nutritional value, texture and flavour due to activity of different kinds of microbes e.g. bacteria, fungi and yeasts. Also, food can play a major role in the transmission of foodborne pathogens such as *Salmonella, Listeria, Campylobacter, E. coli* or cause intoxication such as botulism and staphylococcal food poisoning. Several methods e.g. heating, refrigeration, freezing, drying, chemical preservation, smoking, irradiation and combinations of these have been used to protect foodstuffs against spoilers and pathogens. Despite all of these methods that have been developed over the centuries, some of which are very old, and there is still an urgent need to develop more effective decontamination techniques.

In this thesis, the focus of interest will be on the problems of spoilage and its prevention in relation to seafoods, which are amongst the most perishable of all foodstuffs. Every year, millions of tonnes of fish and fishery products enter into national trade. Seventy percent of the world's catch of fish and fishery products are consumed as food. Finfish and shellfish, after meat and poultry, are the most important animal protein foods for most of the world and consumption of fish is increasing every year. The microbiological condition of this food can be a concern. Fish flesh contains a low level of carbohydrate and high levels of protein and free-non-protein nitrogen (NPN) compounds which, post-mortem, are available to support the growth of spoilage organisms and pathogens. These organisms can reduce the shelf life of the products or threaten the health of the consumers and so reduction in their numbers during processing or packaging and before storage plays an important role in making food safe and providing a longer shelf life.

1.2 Natural micro-flora of fish and shellfish

The population and composition of micro-flora (mainly bacteria) found on the skin, gills and in the intestines of fish after capture is variable and depends on the environment from which they are taken, the season, water quality and conditions of harvesting, handling and processing. Water quality is the most important environmental factor affecting the initial number and types of bacteria. Incidence percentages of different bacteria on the outer surface of newly caught fish and shellfish are shown in **Table 1-1**.

Bacteria from the genera: *Acinetobacter, Cytophaga, Flavobacterium, Moraxella, Pseudomonas, Shewanella* (formerly *Alteromonas*) and *Vibrio* predominate on the surface of fish and shellfish taken from temperate waters, while Bacillus spp., coryneforms and Micrococcus spp. frequently predominate on fish taken from subtropical and tropical waters (115, 171). The Gram-negative bacteria on warm-water finfish are similar to those on cold-water fish. Fresh-water fish show similar patterns except that *Aeromonas* replaces *Vibrio*. *Psychrobacter, Acinetobacter, Corynebacterium* and *Micrococcus* dominate on crustaceans, with a lesser proportion of *Pseudomonas* (153). The composition of the micro-flora of fish from fresh-water environments is also influenced by temperature and will vary from that in marine environments. Method of harvesting, handling and storage of fish in the fishing vessel will also affect the bacterial population. The microbiological quality of brine water and ice, which are used for storage of fish before processing, is a concern, as is the variation in temperature of the fish (171).

The natural bacterial flora on farmed fish and shellfish from temperate waters is similar to the micro-flora of wild fish (177). Those in ponds or in shallow waters near shore are closer to human waste than wild fish, so this may cause a higher frequency of contamination with bacteria that are non-indigenous to the water. For example, *Listeria monocytogenes* was not isolated from unpolluted ocean waters and spring water, but the organism could frequently be isolated from surface waters and polluted sea-water (88), and is found on raw fish e.g. salmon and on products that do not receive a listericidal treatment, e.g., cold-smoked salmon (15, 88). Gram (62) estimated that very high numbers of *Enterobacteriacae* could be found on fish caught in polluted warm waters. Several authors showed that 10-30% of total numbers of bacteria from tropical waters could be Gram-positive including *Bacillus* and *Micrococcus* (12, 92, 170, 185). In some

countries in Southeast Asia, farmers are using human and animal excreta for organic fertilisation or enrichment of shrimp and fish pounds (140, 205). Although with farmed fish and shellfish in tropical waters, the micro-flora is similar to tropical wild fish and shellfish (21, 224), the level of faecal contaminants such as *Salmonella* and *E. coli* are high **(Table 1-3)**. Marine vibrios are also found on shellfish and fish reared in tropical waters (140, 152). A comparison of the surface micro-flora of fish and shellfish from temperate and tropical waters are listed in **Tables 1-2** and **1-3,** respectively.

1.3 Spoilage of fish and shellfish

After death, the flavour and odour of fresh fish will change, due to endogenous biochemical changes in carbohydrates, nucleotides and lipids. Some bacteria change trimethylamine oxide (TMAO) to trimethylamine (TMA), and cause oxidative deamination of amino acids and peptides to ammonia, release of fatty acids, and breakdown of sulphur-containing amino acids to methylmercaptan, dimethyl sulphide and hydrogen sulphide (61, 105). These changes cause the fishy, ammonia and sulphide odours and pulpy texture of spoiled fish. The most common bacteria identified with spoilage are species of *Shewanella* and *Pseudomonas* (94, 115), with *Shewanella putrefaciens* predominating at lower storage temperatures (61, 105). Gram-negative bacteria are dominant on fish spoiled at elevated temperatures (10-37°C), with *Aeromonas* (particularly *Aeromonas hydrophila*), *Vibrio* and possibly coliform bacteria being identified more frequently than *S. putrefaciens*. Indeed, there is evidence that *Aeromonas* or other members of the Vibrionaceae may dominate the spoilage micro-flora of fish held above 5°C (14, 65, 114, 218). Contamination of fish due to contact with nets, ropes, deck boards and human hands or during packing and storing operations below decks can also affect subsequent spoilage.

Shrimp spoilage is different since the animals die immediately after capture. The trawl picks up a huge amount of mud with the shrimp. Bacteria from the mud, ice and boat surfaces can grow during the several days before the shrimp reach the processing plants. Most shrimp have high bacterial counts (10^5-10^7 cfu/g) at the time of receipt at the processing plant. Refrigerated storage selects for a psychrotrophic micro-flora; the

dominant spoilage bacteria appear to be members of the *Acinetobacter-Moraxella* group. However, *Pseudomonas* and coryneform bacteria are commonly associated with the spoilage micro-flora (153).

1.3.1 Spoilage of fish and shellfish stored at ambient temperatures

During ambient storage of tropical fish and shellfish, mesophilic bacteria will reach a level of 10^7-10^9 cfu/g after 12-24 hours (55, 64, 65). The micro-flora is dominated by meshophilic *Vibrio* or *Aeromonas* spp. (59, 66, 169), and, particularly if the fish are caught in polluted waters, mesophilic *Enterobacteriaceae* (62). At ambient temperatures, motile aeromonads are the specific spoilers of aerobically stored fresh-water fish (9, 59, 66). *S. putrefaciens* may also take part in the spoilage (13).

Table 1-1 Bacterial flora on the surface of newly caught fish and shellfish from tropical and temperate marine and fresh waters.

Species	Incidence (%)			
	Temperate		Tropical	
	Marine	Fresh	Marine	Fresh
Gram-negative				
Pseudomonas	0-70	0-22	0-53	0-16
Moraxella		0-14	0-52	0-36
Acinetobacter		0-11	0-15	0-8
"Acromobacter"[a]	5-50	0-10	0-15	0-19
Alcaligenes			0-10	0-10
Flavobacterium	2-25	0-6	0-54	0-13
Vibrio[b]	0-60		0-80	
Aeromonas		0-30		0-2
Enterobacteriace[c]		0-18	0-10	
Chromobacterium			0-20	
Gram-positive				
Micrococcus	0-53	0-10	0-60	0-30
Staphylococcus			0-41	0-18
Bacillus[d]	0-24		0-42	0-5
Coryneforms	0-10	0-12	0-55	0-5
Lactic acid bacteria			0-3	

a) "Acromobacter" denotes Gram-negative, nonfermentative rods, and probably covers strains now identified as *Acinetobacter* and *Moraxella*, and includes *Shewanella putrefaciens*, which is found in low numbers on newly caught fish.

b) Includes *Photobacterium phosphoreum*.

c) In clean waters, mainly psychrotrophic strains (e.g., *Serratia liquefaciens* or *Hafnia alvei*)

d) Clostridia may be isolated in low numbers.

Data from (63)

Table 1-2. Surface micro-flora of fish and shellfish from temperate waters

Wild fish and shellfish	Farmed fish and shellfish
Pseudomonas *Moraxella* *Acinetobacter* *Alcaligenes* *Shewanella putrefaciens* *Flavobacterium* *Vibrio spp.* *Photobacterium spp.* *Aeromonas spp.* *Bacillus spp.* *Micrococcus spp.* *Clostridium* *Corynebacterium*	**Natural bacteria flora is similar to the micro-flora of wild fish and shellfish** + ***Listeria monocytogenes*** ***C. botulinum***

Data from (63)

Table 1-3 Surface micro-flora of fish and shellfish from tropical waters

Wild fish and shellfish	Farmed fish and shellfish
Micro-flora of temperate wild fish + *Enterobacteriaceae* (in polluted waters) *Bacillus* *Micrococcus* *V. cholerae* *V. vulnificus* *V.parahaemolyticus*	**Micro-flora of tropical wild fish** + *Salmonella* (in some Asian countries) *E. coli*

Data from (63)

1.3.2 Spoilage of fish and shellfish stored in ice

During storage of fish, surface bacteria can invade the flesh by moving between the muscles especially when the skin flora increases above 10^6cfu/ cm (160). During ice storage, the aerobic count increases with a doubling time of approximately 24 hours and will, after 2 to 3 weeks, reach numbers of 10^8-10^9 cfu/g flesh or /cm^2 skin (63). The composition of the micro-flora changes dramatically during storage. Thus, during aerobic iced storage, the micro-flora is composed almost exclusively of *Pseudomonas spp.* and *S. putrefaciens*. This is true for all fish and shellfish whether caught or harvested in temperate (65, 109), or subtropical and tropical waters (26, 106, 112, 169).

S. putrefaciens is very important as a spoilage bacterium of iced fish. The bacterium is the specific spoilage bacterium of marine temperate water fish stored aerobically in ice. The number of *S. putrefaciens* is inversely linearly related to the remaining shelf life of iced cod (93). Barile (13) reported mackerel spoilage after 15 days in ice due to *S. putrefaciens* and *Pseudomonas spp.* when iced immediately after capture. However, when held for 9 hours at 26°C before icing, the mackerel spoiled after 5 days due to *Peusodomonas* and mesophilic *Bacillus spp.*. *S. putrefaciens* usually constitutes 1% or less of the micro-flora of fresh fish (23), but increases relative to the aerobic count and constitutes 30% to 90% of the micro-flora at the point of spoilage (23, 93). This bacterium has been isolated from tropical fresh waters, but does not appear to be important in the spoilage of iced fresh water fish from tropical waters (66, 112). *Pseudomonas spp.* are, together with the above bacterium, spoilers of marine tropical fish stored in ice.(26, 55, 62). *Pseudomonas spp.* are also the specific spoilers of iced stored tropical fresh water fish (66, 112). In contrast to *Pseudomonas spp.*, *S. putrefaciens* can grows to levels of 10^5-10^8 cfu/g after vacuum packing and subsequent iced storage of fish from temperate marine waters, because the bacterium is capable of anaerobic respiration using TMAO as electron acceptor (34, 93). *Photobacterium phosphoreum* is another bacterium that has an important role in spoiling of vacuum-packed fish from temperate marine waters (34).

1.4 Characteristics of some spoilage bacteria

1.4.1 Shewanella putrefaciens

The bacterium was first identified as a member of the group *Achromobacter*. This group contained various Gram-negative, non-fermentative, oxidase-positive, rod-shaped bacteria. *S. putrefaciens* then was transferred to *Pseudomonas* by Long and Hammer in 1941. In 1985, MacDonnel and Colwell, suggested that the bacterium be transferred to a completely new species, *Shewanella* in honour of Dr. J. Shewan. The bacterium is a rod motile by polar flagella and has been isolated from marine and fresh water, lakes, sediments, oil fields and proteinaceous foods. The importance of the bacterium in the food industry is due to the spoiling ability of the bacterium in low-temperature stored foods, mostly with high protein content and high pH. So, typically, marine fish, chicken and high-pH meat can be spoiled by *S. putrefaciens*. The organism can change TMAO to TMA and produces a variety of volatile sulphides, including H_2S, which can make a fishy smell. The food spoilage strains of *S. putrefaciens* are all psychrotrophic and grow at 4°C, and many at 0°C. The bacterium rarely grows at 37°C.

1.4.2 Pseudomonas fragi

Members of this genus are Gram-negative, aerobic and straight or slightly curved rods. They are very common in fresh foods because of their association with water, soil and vegetation and they can contaminate meat, milk, poultry, eggs, seafood and vegetables. Many species are psychrotrophic and are important spoilage agents in refrigerated foods. *P. fragi* is oxidase-positive, 0.5-1.0 μm in width x 0.8-4.0 μm in length with a single, polar flagellum and can grow at 4°C but not at 41°C. The bacterium produces lipase, protease and amylase and potentially can spoil milk, seafood and meat. It is important to know that foods spoiled by this bacterium are not harmful for consumers, but they have a lower quality and, due to changes of flavour, odour and texture, may not be consumed.

1.4.3 Micrococcus luteus

The genus *Micrococcus* is Gram positive, spherical in shape with a diameter of 0.5-2.0 µm, non-sporing and usually non-motile and. All species are catalase and oxidase positive and can grow in the presence of up to 5 % NaCl. The primary natural habitat is mammalian skin; the secondary habitat is meat and dairy products, soil and water. It is non-pathogenic, but some strains may be opportunistic pathogens. The cell wall of micrococci consists of a thick, rigid layer of peptidoglycan. *M. luteus* are spheres 0.9 - 1.8 µm in diameter occurring in tetrads and in irregular clusters of tetrads. Colonies are yellow, yellowish green or orange pigmented. The bacterium frequently has been isolated from spoiled fish.

1.5 Bacteria pathogenic for humans and associated with fish and shellfish

Some of the pathogenic agents responsible for health hazards of sea-foods are listed in **Table 1-4**.

Table 1-4 Bacterial health hazards associated with fish and shellfish products.

Indigenous		Non-indigenous	
Toxin preformed in product	**Infection**	**Toxin preformed in product**	**Infection**
Clostridium botulinum (non-proteolytic types B, E, and F)	*Listeria monocytogenes*[a] *Vibrio cholerae* *Vibrio parahaemolyticus* *Vibrio vulnificus* *Aeromonas hydrophila* *Plesiomonas shigelloides* *Shewanella alga*[b]	*Staphylococcus aureus* *Clostridium botulinum* (proteolytic types A and B[c]) Mesophilic histamine-producing bacteria *(Morganella morganii)*	*Salmonella spp.* *Shigella spp.* *E. coli*

a) *L. monocytogenes* seems to persist in the aquatic environment once introduced.
b) *S. alga* is a recently defined species that has not been implicated in foodborne disease but has caused wound infection and bacteremia through contact with warm water.
c) *Clostridium botulinum* types A and B occur in the general environment and are frequently found in water.

Data from (63)

1.5.1 Vibrio cholerae

This bacterium is responsible for epidemic cholera. This disease is an acute, diarrhoeal illness caused by infection of the intestine. Although medical and public health has improved, this disease remains a major public health problem, particularly in developing countries. As a result of the multiple sources of contamination, a wide variety of foods have been directly or indirectly implicated as a vehicle of *V. cholerae,* the most common being fish, shellfish and crustaceans (134). Fish and shellfish may be contaminated with *V. cholerae* at harvest. Marine animals are contaminated both externally and/or in their gut through filter feeding and ingestion (41).

V. cholerae was for many years the only species of the genus of *Vibrio*. The number of species within the genus has increased considerably during the last 30 years and currently numbers more than 30. The species is divided into serotypes based on their O somatic antigens. Strains associated with cholera have typically possessed the O1 antigen, although non-O1 serotypes are now also responsible for cholera-like disease. In 1991, long-distance translocation of the bacterium was demonstrated when *V. cholerae* O1 was isolated from shellfish harvested off the US Gulf (39, 129). The isolated bacterium were shown by molecular typing to belong to the same clone as Latin American strains prevalent at the time. Further investigations revealed that bilge and ballast water on American ships present in the area were contaminated with the bacterium (129). Isolation and identification of *V. cholerae* non-O1 from oysters, different fish species, environmental samples, seafood and patients after eating seafood have been reported (120, 128, 163).

1.5.2 Vibrio parahaemolyticus

The organism has been isolated from fish, shellfish and other seafood and also from coastal waters. *V. parahaemolyticus* is frequently isolated from frozen, cooked seafood from Eastern countries. Due to its halophilic nature and the marine source of *V. parahaemolyticus*, raw and cooked seafood such as shrimp, prawn, lobster and crab may carry the infection. In Western countries, raw molluscs and cooked crustaceans are the most common food source of *V. parahaemolyticus* while, in Asian countries, fish is a

common source because it is eaten raw. Although *V. parahaemolyticus* is sensitive to heat, cooked foods may be contaminated by raw products with which they come in contact. The numbers of organisms present in naturally contaminated food is low, approx. 100 g^{-1}, and may increase ten-fold in the summer (40). Thus an increase in number during storage is usually necessary to establish an infection in healthy hosts.

V. parahaemolyticus infections occur worldwide but most of the food-borne disease outbreaks of the organism due to seafood have occurred in Asian countries with the highest incidence in Japan (95). In 1999, during a study of samples of seafood imported from Hong Kong, Indonesia, Thailand and Vietnam, *V. parahaemolyticus* was recovered from 45.9% of samples. The incidence rates were: for shrimp 75.8%, crab 73.3%, snail 44.3%, lobster 44.1%, sand crab 32.5%, fish 29.3% and crawfish 21.1% (217). Among 622 outbreaks of food-borne illness reported in Taiwan during 1981 to 1989, the most frequently isolated organism was *V. parahaemolyticus* and seafood products were the major source (27). In another study, among 102 outbreaks of food borne disease reported to the Taiwan Department of Health, it was responsible for 56.7%. This organism has been a leading cause of problems, particularly in the warmer months, in Taiwan for many years (145), frequently in food from sea water and fresh water (218). It has also been found, with an incidence of 77%, in oysters (*Crassosterea gigas*) originating from the southern coast of the state of Sao Paulo, Brazil (128).

Generally, the incidence of *V. parahaemolyticus* gastro-enteritis is usually higher in the summer following the seasonal pattern of the bacterium in the natural environment. Naturally contaminated seafood e.g. fish, shellfish and crustaceans are the major source of the pathogen, either eaten raw, inadequately cooked or cross-contaminated after cooking (90).

1.5.3 Vibrio vulnificus

The Centre for Disease Control reported a role of *V. vulnificus* in food-related disease in 1976 (17). Seawater and aquatic creatures were the main habitat of this organism (38, 143). It can cause wound infections, gastro-enteritis, or a syndrome known as "primary

septicaemia" (U.S. Food and Drug Administration, http://vm.cfsan.fda.gov/~mow/chap10.html). *V. vulnificus* is a mildly halophilic bacterium that occurs naturally in estuarine and seawaters, residing in high numbers in filter-feeding shellfish such as oysters, clams and mussels. It has also been isolated from a wide range of environmental sources, including water, sediment, plankton and other marine fish and crustaceans in a variety of countries (148). The bacterium infects only humans and other primates but most healthy people are resistant to infection. A strong association was seen between *V. vulnificus* infection and patients who had certain underlying diseases such as liver dysfunction, certain blood disorders, diabetes, cancer, increased serum iron levels with chronic alcohol abuse and with malignancies or gastrectomies (28, 90, 137, 148, 191). No major outbreaks of illness have been attributed to this organism. Sporadic cases occur frequently, becoming more prevalent during the warmer months and with a high mortality rate of between 40 to 60% (90).

1.5.4 Clostridium botulinum

Clostridium botulinum, one of the most important of the pathogenic food-borne bacteria, is an anaerobic, Gram-positive, spore-forming rod that produces a potent neurotoxin and it is responsible for the paralysing disease botulism. This species is divided into seven types (A, B, C, D, E, F and G) on the basis of the antigenic specificity of the toxin produced by each strain. All of these strains produce neurotoxins with similar effects on the host, but the different types of toxins are serologically distinct. Types A, B, E and F cause human botulism whereas types C and D cause most cases of botulism in animals. Types A and B are found primarily in soil and seawater sediments and in fish or invertebrates in fresh water and seawater. Type E outbreaks are usually associated with consumption of fish (22, 192, 214). The organism has been isolated from fish farms and farmed trout (19), waterfowl (144), the fresh water environment (198) and in fish from markets (74).

Generally, any food that, when processed, allows spore survival, and is conducive to outgrowth of spores and toxin production, and is not subsequently heated before consumption, can be associated with botulism.

1.5.5 Salmonella

Salmonellae are Gram-negative, rod-shaped and non-spore forming, usually 0.7-1.5×2-5 μm in dimensions and the majority of them are motile by peritrichous flagella. At present, more than 2370 specific serological types are recognised. Salmonellae are frequently found in the intestinal tracts of domestic or wild animals. They can survive for 10-12 weeks in water and for many months in faeces, soil and pasture (Josland, 1951; Mair and Ross, 1960). Surface waters can be contaminated by run-off from farms and from sewage.

Seafood can be contaminated with salmonellae in rivers, lakes and in-shore waters, or during handling after harvesting. Food wastes and animal manures are often fed to fish and crustaceans in earthen ponds and salmonellae are frequently found in the ponds. Brackish water and shrimp raised in them are inherently contaminated with salmonellae and, at higher stocking densities, the incidence of salmonellae increases (152). Food animal origins are the primary vehicles for outbreaks of the disease. Fish and shellfish have been responsible for two salmonella outbreaks in Canada in 1982, five in England and Wales in 1984, four in Poland in 1980 and one in the USA in 1982 (35).

Farmed seafood, or seafood harvested from in-shore waters or rivers may contain salmonellae and fish caught in deep waters may be contaminated after harvesting. Among 211 shrimp samples from various countries, 8.1% were positive for salmonellae (54) and among 494 samples of catfish in the USA, 5.2% were positive (36). In another study, salmonellae were isolated from smoked fish and shellfish (78).

1.5.6 Listeria monocytogenes

Listeria monocytogenes is a food-borne pathogen, which can cause outbreaks and sporadic listeriosis. The organism has been found in at least 37 mammalian species, both domestic and feral, as well as 17 avian species and some species of fish and shellfish. Some studies suggest that 1-10% of humans may be intestinal carriers of the organism. It

is also isolated from soil, silage and other environmental sources. (U.S. Food and Drug Administration, http; /vm.cfsan.fda.gov/~mow/chap6.html).

1.5.6.1 Taxonomy

The organism was first described in 1926, after an infectious epidemic among laboratory rabbits and guinea pigs (139). The isolated organism was named *Bacterium monocytogenes* because the infection was characterised by a monocytosis. A similar bacterium isolated from the liver of infected gerbils was named *Listeralla hepatolytica* by Pirie (151), who suggested the current name Listeria in 1940 in honour of Lord Lister, an eminent English surgeon. Now, *L. monocytogenes* is situated in the genus *Listeria* and it is generally accepted that the genus consists of 4 species and 3 subspecies (186).

1.5.6.2 Bacteriology

Cells are Gram-negative, short, regular rods with rounded ends that can occur singly, in parallel or in short chains arranged to form a V shape. The cells are 0.4-0.5μm in diameter and 0.5-2.0 μm in length. The organism is motile by means of a few peritrichous flagella when grown at 20-25°C. *Listeria* spp. will grow in most bacterial culture media but growth is enhanced in the presence of glucose, serum and blood. After incubation for 24h at 37° C on nutrient agar, colonies are 0.5-1.5 mm in diameter, round, translucent with a watery appearance and have a finely textured surface and an entire margin (123).

1.5.6.3 Pathogenicity

Among *Listeria* spp., *L. monocytogenes* and *Listeria ivanovani* are pathogenic to humans and animals. Listeriosis, the disease caused by pathogenic listeria, can affect many of the body's organs, including the gastrointestinal tract. Immunosuppressed humans are more likely to become ill but many people will remain symptomless. It is hypothesised that isolation of *Listeria* spp. from human stools may merely reflect the transit of the organism from contamination within a given food. Detection methods to

distinguish between virulent and non-virulent strains of *L. monocytogenes* are still being developed so those used are based on historically accepted techniques (186). It has been reported that β-haemolysin produced by *L. monocytogenes* is correlated to the pathogenicity of the organism (45), also a number of specific genes play a role in virulence of the bacterium. Genes essential for intracellular replication and intra and intercellular mobility are located in a chromosomal region between *ldh* (encoding lactate dehydrogenase) and *prs* (encoding phosphoribosyl-pyrophosphate synthetase) genes. The extracellular protein p60, which has peptidoglycan hydrolase activity and catalase, superoxide dismutase and also a group of genes, which encoding large and small internalins have been recognised to be involved in pathogenicity (57).

1.5.6.4 Ecology

Isolation of listeria has been reported from both cultivated and uncultivated soils, mud and moist soils (68, 215) as well as from surface and spring water (67, 211) and sewage (5, 211). Because the organism is isolated from surface waters, it was suggested that waters receiving sewage effluent may be a route for recycling listeria (43, 211). Such waters were contaminated up to a distance of 25 miles from a treatment plant (43). *L. monocytogenes* has been isolated in dairies (187), in poultry processing (10) and in meat processing factories (194). In seafood processing environments, *L. monocytogenes* is isolated more frequently from chiller rooms, as well as from floors and drains. Brine tanks were also identified as a source of listeria contamination (186).

1.5.6.5 Epidemiology

L. monocytogenes is a food-borne pathogen that is capable of causing sporadic and epidemic illness. Although some believe that humans are infected after direct contact with diseased animals (195) and soil, *L. monocytogenes* is an environmental contaminant and probably the primary means of transmission to humans is through food contaminated during production and processing (6).

The well-categorised risk groups include pregnant women and their foetuses, neonates, the elderly, and adults with a compromised immune system, e.g. renal transplant patients, patients on corticosteroid treatment, HIV/AIDS patients and alcoholics, whose resistance to infection is low (2, 4, 25).

Molecular typing technology has improved the epidemiology of listeria. The most common serotypes isolated from food-borne listeriosis are 4b, 1/2a and 1/2b (186), although not all *L. monocytogenes* isolated from food are pathogenic (80, 154).

The minimum infective dose for food-borne listeriosis is still not defined; however, the most at-risk foods are reported to be ready-to-eat meals, requiring no further heat treatment. These include ready-cooked chicken, sliced ham, pate and processed meat paste, shellfish products, soft and surface ripened cheese and foods held under refrigeration (73, 154).

Surveys of fresh water, sea water and live fish and shellfish have suggested that contamination with *Listeria spp.* is more likely to occur in fresh water fish than sea water, and is dependent on the presence of the bacteria in the surrounding waters (15). *Listeria* species (81%) and *L. monocytogenes* (62%) were found in a high percentage of samples of fresh water (river, contact with domestic animals) in a Californian coast estuarine environment (29).

1.5.6.6 Prevalence of *L. monocytogenes* in the fish industry

L. monocytogenes can grow at refrigeration temperatures of 4° C and down to 1° C (166). Heating at 60° C should achieve a 4-log reduction over various heating times depending on the level of curing salts used and the fat level of the fish (15).

Prevalence of *L.* monocytogenes in fresh, frozen and processed seafood has been well investigated. The organism has been found in frozen fish (18), frozen minced fish (158), raw seafood (161) and frozen and processed seafood (76). Also *Listeria spp.* and *L. monocytogenes* were found in raw, cooked, processed and frozen shrimp (15, 122, 127).

Survival of the organism in smoking and other light preservation processes like marinating and curing is of concern (71, 91, 121). *L. monocytogenes* has frequently been

isolated from cold-smoked salmon in different countries. Rorvick and Yndestad (158) reported *L. monocytogenes* in 9% of cold-smoked salmon samples in Norway whereas Farber (48) reported 31.2% of samples positive for the bacterium in Canada and Hudson *et al.* (86) reported 75% positive in New Zealand. Some believe that the organism cannot survive after hot-smoking process carried out at 65°C for 20 min (91), but Dillon *et al.* (44) reported isolation of *Listeria spp.* (25.4%) from hot-smoked fish. However, this may have been due to post-processing contamination. The contamination of salmon by *L. monocytogenes* during cold-smoking processing is a concern. A high incidence of contamination with *L. monocytogenes* has been reported during filleting and the subsequent smoking processing. Although the incidence was increased on fillets and smokehouse environment samples, no fillets were positive immediately after smoking and before further processing, suggesting that the cold smoking process had an effect on reducing the numbers of the bacterium. The authors emphasized that further investigation was required in this area (157). In another study, two cold-smoked salmon processing plant were studied. In the first processing plant, no *L. monocytogenes* was found on raw fish but the level of final product contamination varied between 31-85% whereas, in the second processing plant, the level of contamination of both raw fish and the product varied from 0 to 25%. It was concluded that contamination of the final product (cold-smoked salmon) was due to contamination during processing rather than to contamination from the raw fish. However, the possibility that raw fish was an important source of contamination of the processing equipment and environment could not be excluded (201).

Although no cases of listeriosis have so far been directly linked to smoked-salmon, the presence of *L. monocytogenes* is a serious concern due to the fact that product is consumed as ready-to-eat products without a heat treatment, which would inactivate the pathogenic bacteria. In a study to evaluate the *L. monocytogenes* subtypes associated with foods, specifically smoked fish, the data suggested that at least some of the subtypes present in ready-to-eat foods may have only limited human-pathogenic potential (141). Isolation of *L.* monocytogenes from food may be complicated. There is little quantitative work on the levels of *L. monocytogenes* in seafood products due to the fact that the bacteria may have been sub lethally injured by heating, freezing, acidification or drying

and use of pre-enrichment broths are necessary for recovery of the injured cells. Reported levels have been approximately 100 cfu/g (133).

1.5.6.7 Isolation and enumeration

Based on different types of food, several methods of isolation have been used. The two common isolation protocols, which also have been used for detection of *Listeria spp.* in seafood, are those formulated by the US Food and Drug Administration (190) and by the US Department of Agriculture (130). The most common selective enrichment broths, which have been used in both methods and their modifications are: Listeria Enrichment Broth (LEB), Fraser Broth, Polymyxin Acrifavin Lithium Chloride Ceflazidime Aesculin Mannitol Egg Yolk Broth (PALCAM) and the most common isolation plating media used are Lithium Chloride Phenylethanol Moxalactam (LPM) medium, Oxford agar, modified Oxford agar, PALCAM agar and Haemolytic-Ceftazidime-Lithium Chloride agar (HCLA).

Lovett *et al.* (118) compared the FDA and the USDA method for the recovery of *L. monocytogenes* in inoculated seafood and reported that the FDA method is better for isolation of heat-stressed cells and the USDA method may a better procedure for recovery of unheated *Listeria* from a high background. In 1994, Ben Embarek (15) suggested that PALCAM agar or modified Oxford Agar are superior for products such as smoked salmon with non-stressed *Listeria* cells and a large background flora. The use of direct plating for recovery of *Listeria*, expected to be injured or stressed in most seafood was not recommended (16). In a comparison of selective direct plating media for recovery and enumeration of *L. monocytogenes* from artificially-contaminated cold-smoked salmon, Oxford agar and Lee's modification of Oxford agar, in comparison to other agars were satisfactory (147).

1.6 Preservation of sea-foods

Several ways have been used for preservation of fish and shellfish including:

1.6.1 Chilled products

Storage in ice is the general method. This method is normally used to protect fish and shellfish from spoilage as much as possible during transport to the processing plant to ensure both microbiological quality and safety. The periods involved vary from a few hours to 3 weeks or more. Storage is normally in melting ice or chilled brine (or sea water) at -2°C. Important bacteria in fresh and packed fish and shellfish stored chilled or in ice are shown in **Table 1-5.**

1.6.2 Frozen products

In most cases seafood are frozen unwrapped to facilitate rapid freezing, but for some purposes products may be packaged before freezing. All types of freezing systems are used for seafood including contact plate or shelves, brine and other direct contact refrigerant systems, continuous moving-belt air-freezing systems and passive air blast freezing as well as traditional sharp freezers (150). Frozen seafood is taken to a temperature below –18°C and, more commonly with modern practices, to even lower temperatures. Storage of frozen seafood is at -20°C or lower to maintain product quality. Fish frozen before rigor mortis are often held at -7°C for a few days to enhance quality.

1.6.3 Lightly-preserved seafood products

This group includes fish products preserved by a low level of salt (<6% [w/w] NaCl in the water base) and, for some products addition of preservative such as sorbate, benzoate, NO_2^- or smoke. The pH of the products is high (>5.0), and they are often packaged under vacuum and must be stored and distributed at chill temperature (≤5°C). The products usually have a shelf life of 3 to 6 weeks. These products are the most dangerous group and almost any of the pathogenic organisms listed in **Table 1-4** may be transferred via these types of stored food and distributed at chill temperature (≤5°C). *C. botulinum, L. monocytogenes* and *Aeromonas spp.* can grow in these products and spoilage is mainly caused by bacterial action. Bacteria important in lightly preserved seafood are shown in **Table 1-6.**

1.6.4 Semi-preserved seafood products

Fish products with a high salt content (>6% NaCl in the water phase) or pH below 5.0 to which preservative (benzoate, sorbate, nitrate) may be added, are defined as semi-preserved. Several kinds of products from Northern Europe, Southern Europe and Southeast Asia are semi-preserved. Raw material for caviar production is a semi-preserved product and is held in 4% to 5% acetic acid and 5% to 10% NaCl for 2 to 4 weeks. Storage temperature is ≤10°C. *C. botulinum* may constitute a serious risk in these products and spoilage is caused by the activity of the lactic acid micro-flora or by autolytic changes. Important bacteria in semi-preserved seafood products are shown in **Table 1-7.**

1.6.5 Heat-treated seafood products

Many seafood products receive a heat treatment as part of the processing. Depending on the temperature used, they can be classified into the following four categories:

1. Pasteurised products that are often vacuum packed and must be distributed at chill temperature (5°C), such as hot-smoked fish, which is usually brined or dry salted and dried before smoking at an internal temperature of approximately 65°C for 45 minutes.

2. Cooked products, e.g., shrimp or cooked and breaded fish fillets, often packed and sold frozen.

3. Canned (not sterilized) products, e.g. crab meat, these products, which receive heat treatment for 1 min at 77.2°C – 98.8°C, must be distributed at chill temperature.

4. Commercially sterilized (canned) products: These products including canned mackerel, tuna and salmon have received sufficient heat treatment to allow distribution and storage at ambient temperature.

Bacteria important in heat-treated seafood products are shown in **Table 1-8.**

1.7 Some methods which could potentially be used for preservation of food

Some methods, which are already in use for food prevention but have not necessarily been widely implemented, and other methods with potential for food preservation are described below:

Table 1-5 Important bacteria in fresh and packed fish and shellfish stored chilled or in ice

Spoilage bacteria	Health hazards
Pseudomonas spp. *Shewanella putrefaciens*	*C. botulinum* *V. parahaemolyticus* *V. vulnificus* *V. cholerae* *A. hydrophila* *Y. enterocolitica* *L. monocytogenes*

Table 1-6 Important bacteria in lightly-preserved seafood products (<6% NaCl + preservative)

Spoilage bacteria	Health hazards
Lactic acid bacteria *Hafnia alvei* *Serratia liqefaciens* *Enterobacter spp.*	*C. botulinum* *L. monocytogenes* *Aeromonas spp.*

Table 1-7 Important bacteria in semi-preserved seafood products (> 6% NaCl + preservative)

Spoilage bacteria	Health hazards
Halococcus spp. *Lactic acid spp.*	*C. botulinum*

Table 1-8 Important bacteria in heat-treated seafood products

Spoilage bacteria	Health hazards
Heat-resistant psychotrophic Molds and yeast	*C. botulinum* *L. monocytogenes* *B. cereus*

All above data from (63)

1.7.1 Radiation

These techniques rely on the direct action of the radiation on the microbes and can be classed into two types, ionising radiation and UV radiation.

1.7.1.1 Ionising radiation

Ionising radiation includes gamma radiation, X rays and accelerated electrons. Gamma radiation is not a new technology and has been used for many years for the sterilisation of medical supplies and for the treatment of plastics to reduce contamination. The use of the technology for treatment of food is also not new. Today, many countries use this

technique for processing certain foods such as spices, fruits and vegetables. Two countries have used the irradiation facilities for disinfection of seafood. One is located in France for shrimp and has been in use since 1989 and the other in Bangladesh for dried fish, which has been in operation since 1993 (149).

The biological action of radiation is through the disruption of the main target, DNA. Water in moist foods is ionised by radiation. Electrons are expelled from the water molecules and cause the breakage of chemical bonds. The products then recombine to form hydrogen, hydrogen peroxide, hydrogen radicals, hydroxyl radicals and hydro prolix radicals. These radicals are very short-lived (less than 10^{-5} sec), but still cause sufficient damage and destroy the bacterial cell. The damage to the cell is achieved with radical and ionic attack on the cell wall, membrane and on the cell metabolism. Later, the direct effect of the radiation on the DNA molecule becomes apparent when the helix fails to unwind and therefore the organism cannot reproduce (Ginoza, 1967). The damage to the DNA is due to the production of double-stranded breaks and occasionally thymine dimers (8). Because ionising radiation relies upon the interaction of ions with the target, the smaller and simpler the organism is, the higher the dose of radiation needed to destroy it. Also, in general, Gram-negative bacteria are more sensitive than Gram-positive ones and rods are more sensitive than cocci. Yeast and moulds tend to be more resistant than bacteria, and bacterial spores are even more resistant, viruses are generally the most resistant of the microorganisms (149).

1.7.1.2 UV radiation

Ultraviolet radiation includes the portion of the radiant energy spectrum between visible light and X-rays. UV radiation has a wavelength approximately between 100 and 400 nm. It has been divided by the International Commission on Illumination into three regions UV-A (315 to 400 nm), UV-B (280 to 315 nm) and UV-C (100 to 280 nm). Wavelengths below 100 nm are generally absorbed in the air and this region is also termed the vacuum ultraviolet (VUV) range.

UV radiation is generally effective at reducing bacterial populations (11, 24, 85, 96, 222). The sensitivity of microorganisms to UV radiation varies with the wavelength of

the radiation. Gartner in 1947 reported that the optimum lethal wavelength for all microorganisms was at 254 nm and this wavelength has been used for many bactericidal applications. Many factors may affect the action of UV radiation on bacteria such as: depth of the layer irradiated, starting concentration of the suspension of the test organisms, transmittance at 254 nm of the suspension, type and arrangement of UV lamps, method of dose measurement and irradiation time (175). Like ionising radiation, UV is effective primarily on the cellular DNA. The major damage is against the more sensitive pyrimidine bases (132). The damage is primarily due to the production of linkage between successive pyrimidines on the DNA strand forming dimers, but a minor effect is due to double-strand breaks (8). Cross-linking of the DNA and protein play a significant role in the killing of the cells (173).

Although UV has low penetration power, it is used frequently in order to kill microorganisms that exist on the surface of a variety of materials and in water (79, 167, 174). A comparison study was done on the germicidal effect of UV light on pathogenic and indicator organisms. The doses of UV light necessary for a 99.9% inactivation of cultured vegetative bacteria and total coliforms were comparable. However, to inactivate viruses, bacterial spores and the amoeba cysts, doses required were about 3 to 4 times, 9 times and 15 times, greater respectively (24). In another study, Butler *et al.* (20) found 1.8, 2.7 and 5.0 mWs/cm^2 were required for a 3 log reduction (99.9% inactivation) of *Campylobacter jejuni, Yersinia enterocolitica* and *E. coli* in 1.0 cm depth of bacterial suspension. Sommer *et al.* (176) exposed three *E. coli* strains, three bacterial viruses and spores of *Bacillus subtilis* to UV light. They found that the *E. coli* strains and phage phi X174 were most UV susceptible, followed by phage B40-8 and finally MS2 and bacterial spores. The killing effect of UV also has been investigated on antibiotic-resistant strains of *Staphylococcus aureus* and *Enterococcus faecalis* in order to treat wound infections. Results suggest that Enterococcal bacteria are more susceptible to the killing effect of UV. These data also suggest that UV light at 254 nm is bactericidal for antibiotic-resistant strains of *S. aureus* and *E. faecalis* at times as short as 5 seconds (30). The effect of UV on three bacterial and one viral fish pathogens was investigated in water of different salinities. A UV dose of 2.7 mWs/cm^2 resulted in a 99.999% (5 log) reduction in viable count for *Vibrio anguillarum, Vibrio salmonicida* and *Yersinia ruckeri*. 122

mWs/cm^2 was required for a 3-log reduction of *infectious pancreatic necrosis virus (IPNV)* (111). Straus et al (181) applied high intensity ultraviolet germicidal (UVG) lamps at various times and distances against *L. moncytogenes*. They indicated that UVG irradiation is an effective way of killing the bacterium on surfaces and could therefore be useful in the food manufacturing industry.

UV radiation in food industries

The use of UV radiation as an applicable method for bacterial decontamination of foods has received limited investigation in the last two decades. In 1982, the effect of UV irradiation at 254 nm and doses of 300 mWs/cm^2 from a photochemical reactor or 4.8 Ws/cm^2 from a high intensity UV-C lamp on the microbiological count and storage-life of fish was studied. A 2-3 log reduction in surface microbial count of mackerel was reported. Also, the treated fish wrapped in 1 mm polyethylene and packed in ice at –1°C had a shelf life of at least 7 days longer than that of untreated controls. The results showed that spray washing with chlorinated water by itself or in combination with UV was necessary to reduce surface counts on rough fish to the same extent as those on smooth- surface fish treated by UV alone(85).

Fresh meat has been the subject of other studies. Experimental results showed that UV treatment of fresh beef could effectively increase the lag phase of bacterial growth until adequate cooling had occurred. It was reported that, UV was more effective on the smooth surface of beef, where the meat fibres are parallel to the surface than rough surface cuts of meat such as round steak (180). It was also shown that UV could be used to reduce *E. coli* and *Salmonella senftenberg* on pork meat surfaces. The authors suggested more research was needed to determine the antimicrobial activity of UV exposure of meat carcasses or meat cuts in a food-processing environment (216).

When *Salmonella typhimurium* on agar plates and poultry skin was treated by UV energy, a 3-log (99.9%) reduction in viable count on agar plates was obtained at 2000μWs/cm2, whereas, on the surface of poultry skin, an 80.5% reduction in *Salmonella typhimurium* was obtained with same UV energy (183). Another study on broiler carcasses suggested that UV radiation could reduce *Salmonella* surface

contamination without a negative affect on carcass colour or increasing the rancidity of the meat (203). Also UV was also shown to reduce *Salmonella enteritidis* on agar plates and eggs shell. The dosage of UV was about 6 times more effective on the bacterium on agar plates than on the eggshell (103). Combined treatment with UV, chlorine, sodium chloride and trisodium phosphate on the reduction of *Yersinia enterocolitica* and mesophilic aerobic bacteria on eggshell surfaces was studied by Favier *et al.* (49). They found that, on un-inoculated eggs, the best results were obtained by a combination of chlorine and UV. On *Y. enterocolitica*-inoculated eggs, a combination of trisodium phosphate and chlorine gave the highest reduction. It was concluded that *Y. enterocolitica* was more resistant to UV radiation than the natural mesophilic aerobic micro-flora on the eggshells, except when a low inoculum (4.39 log cfu/egg) was assayed.

The effect of UV on reduction of bacteria in food package cartons is another area that has been studied. A synergistic effect between low concentrations of hydrogen peroxide and UV irradiation on spores of *Bacillus subtilis* was reported. The type of inner surface of the carton was important for the efficiency of the treatment (179).

Effects of UV on food

It is well known that UV irradiation can destroy certain vitamins, particularly vitamin C and B vitamins. UV can also promote the formation of vitamin D from its precursors. The oxidative deterioration of oils and fats, leading to rancidity, has been reported following UV irradiation but there is some evidence to suggest that conducting the irradiation under an inert gas blanket can reduce these effects. Application of UV in foods would be limited to the doses which can be used safely on foodstuffs containing high levels of oils and fats. The intense irradiation of fish oils has been shown to be linked to the production of toxic by-products, e.g. aldehydes, but there have been no reports of the occurrence of these or similar compounds in foods which have undergone UV irradiation (168).

1.1.1 Microwave

Microwaves, in comparison to infrared and UV radiation are relatively long transverse waves of 1 m to 1 mm in wavelength. They cover the broad range of radio frequencies from 300 MHz to 300 GHz. **Figure 1-1** shows the electromagnetic spectrum and the relative position of microwaves.

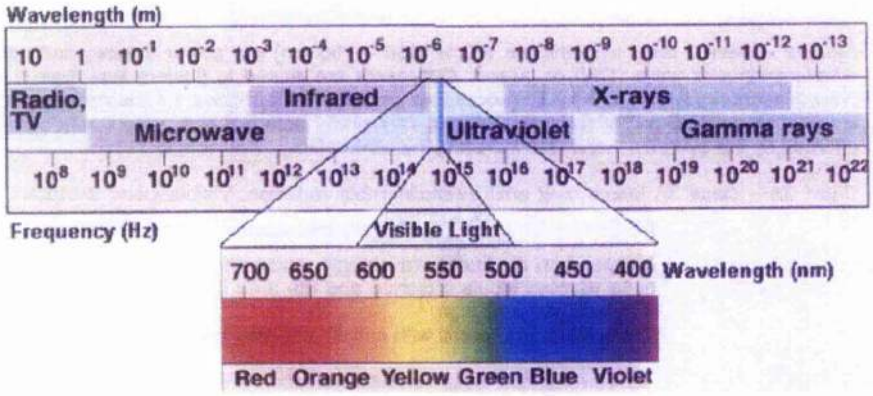


Figure Error! No text of specified style in document.**-1. The electromagnetic spectrum**

Microwaves are reflected by metals, transmitted by electrically neutral materials such as glass, most plastics, ceramics and paper and absorbed by electrically charged materials. The conventional microwave oven normally works with a frequency of 2450 MHz and most of the sterilisation studies on food and destruction of microorganisms reported use this frequency. Microwaves penetrate into food materials and, as they penetrate, the energy they carry is converted to heat, actually by the food material itself, mainly by the mechanisms of polar and ionic orientation (116) **(Figure 1-2).** Culkin and Fung, (32) reported that low frequency-range microwaves have better penetration into food than higher ones. For large industrial applications in some countries, 915 MHz is used for better penetration.

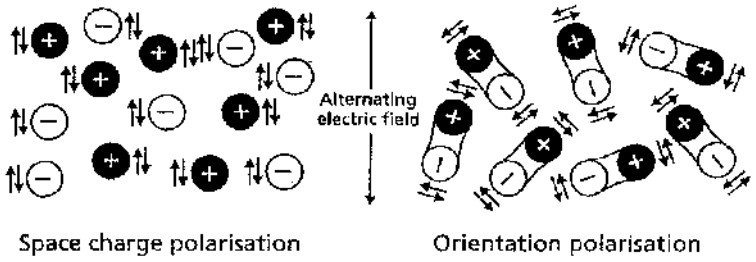


Figure 1-2. Ionic and polar orientation

A few studies have reported that the bactericidal effect of microwave radiation may be due to non-thermal effects. For example, tomato soup, vegetable soup and beef broth were inoculated with *E. coli* and *Salmonella typhimurium* and heated in a 915 MHz microwave oven. Temperature monitoring by temperature-sensitive paper strips showed that different levels of the soups reached different temperatures and also fewer surviving bacteria were found at the top of the container, which had the coolest temperature. Based on these results, the authors believed that an extra effect, more than simple heating caused bacterial killing (32). The effect on the activity of numerous enzymes of *S. aureus* was examined after microwave and conventional heating. The data indicated that activities of some enzymes after the treatments may have been changed. For example, malate dehydrogenase activity increased after conventional heating, but not to the extent found in microwave-treated cells. The ratio of enzyme activity for heat-treated cells was 1.84 versus 2.72 from microwave-treated cells. Also, the activity of ∝-ketoglutarate dehydrogenase decreased with the ratio 0.24 for heat-treated cells versus 1.76 for microwave-treated cells. So, it was reported that microwave heating affected enzyme activity levels in a manner unlike that observed in conventionally-heated cells (46).

Khalil and Villota (100) did a comparison of microwave and conventional heating on the destruction of *Bacillus stearothermophilus* spores. A greater lethality resulted from microwave heating than from conventional heating to the same temperature (212°F). Thus they concluded that there was evidence of an non-thermal effect of the microwave radiation. In another study, several tubes containing 10 ml of suspension of *S. aureus* were subjected to various thermal treatments by microwave and conventional heating. By using kerosine, circulated around the sample tubes situated in microwave cavity, it was tried to continuously remove heat from the sample and keep the set temperature stable. The kerosene was cooled in another heat exchanger outside the microwave cavity using cold water. Some tubes were exposed to conventional heating by distilled water bath. For both heating modes, the toluol thermometer was used to eliminate any variance between the temperature monitoring devices. A greater injury was observed on *S. aureus* by microwave compared to a conventionally-treated cells at the same temperature and condition (99). Sun *et al.* (184) reported that microwave accelerated the rate of phosphoanhydride bond hydrolysis in RNA more than that observed after conventional heating. This contrasts with a report by Rosen (159) that microwave energy is non-ionizing and incapable of breaking chemical bonds. In all of the above studies, it seemed that the investigators had difficulties in temperature monitoring and/or control of temperature.

In contrast, other investigators believe the microwaves reduce bacterial numbers entirely by thermal effects (58, 89, 107, 197). These effects include potentially irreversible heat-denaturation of enzymes, proteins, nucleic acids or other cellular constituents vital to cell metabolism or reproduction, resulting in cellular death (77). However, this does not mean that athermal effects do not occur in biological systems. In the specific area of the interaction of microwaves with microbiological systems, there is very little evidence to support the existence of athermal effects and it seems that this area needs more investigation.

Lin and Sawyer (113) used 2450 MHz microwave radiation on beef and results showed that beef loaf wrapped in polyvinylidene chloride (PVDC) film had lower levels of survival of aerobic bacteria, *S. aureus* and *E. coli* than unwrapped beef loaf. Hollywood *et al.* (83) demonstrated that *L. monocytogenes* survived in all samples of

mince beef cooked by microwave to the rare state, prior to the standing period and was still present in one of three samples after the standing period. *L. monocytogenes* was also detected in one of three samples cooked to medium prior to a standing period. This organism was not detected in mince cooked by the conventional oven method.

Thompson and Thompson (193) and Villamiel *et al.*, (199) demonstrated that continuous microwave processing may be an effective and mild approach for the pasteurisation of milk without changing its organoleptic quality.

The killing effect of microwave on *L. monocytogenes* in chicken has been the subject of some studies. Coote *et al.*, (31) did experiments on chicken skin and whole chicken and showed that when a temperature of 70° C is reached and maintained for at least 2 min throughout a food there is a substantial reduction in the number of *L. monocytogenes*. In another study, survival of the bacterium inoculated within and onto the surface of stuffed chickens was examined. Results showed that although high temperatures (72-85° C) were recorded at various locations in and on the chickens, some viable bacterial were still recovered. Lack of uniform heating within microwave-cooked foods was suggested as a factor and the necessity of enough standing time to allow for temperature equilibration within the food when microbiological safety was important (119). When different strains of *L. monocytogenes* were inoculated into various chicken dishes and other chilled foods before microwave cooking it was found that, even after following the manufacturer's cooking instructions, the temperatures necessary to kill the bacterium were not achieved within some foods (202). In other work, *L. monocytogenes* was surface-inoculated on to chicken breast and the chicken was exposed to the microwave and internal endpoint temperatures of 65.5, 71.7, 73.9, 76.7 and 82.2° C were achieved. The three highest temperatures achieved 2.5 to 3.5 log reductions in viable counts whereas the two lowest temperatures reduced the bacterial population by less than 2 logs. Also it was concluded that the temperature achieved within microwave-heated foods might vary widely. Surface temperature is usually drastically different from the internal temperature and so, due to lack of uniformity of chicken breast samples and differences between surface and internal endpoints, assigning the conditions sufficient to destroy *L. monocytogenes* in poultry would need to be done carefully (75).

In 1995, Gundavarapu *et al.* (70) studied the effect of different microwave power levels on the survival of five strains of *L. monocytogenes* in inoculated shrimp. They macerated one hundred grams of shrimps by a blender and added 1 ml of Listeria suspension containing $2x10^9$ cfu/ml to make a uniform distribution of *Listeria* in shrimp sample. The samples were cooked in the microwave oven at different power levels (240, 400, 560 and 800 W) using cooking times predicted by a mathematical model as well as 20% longer times than those obtained from the model. Samples then were left for two min to allow temperature to equilibrate through the product. Results showed that at least one replication of inoculated shrimps was positive for the presence of *Listeria*. No viable *L. monocytogenes* were detected in shrimp cooked at 120% of predicted times. They found that a mixture of the *Listeria* could be completely inactivated with 2 min holding after microwave treatment for 168, 84, 62 and 48 s at 240, 400, 560 and 800 W, respectively.

1.7.3 Laser

Since its introduction in the early 1960s, laser technology has progressed very rapidly and today lasers are used in many fields such as welding, astronomy and surgery. The term LASER is an acronym for **L**ight **A**mplification by **S**timulated **E**mission of **R**adiation. The amplification will produce a beam of photons with identical scalar and vector properties such as frequency, phase, direction and polarisation to the photon-inducing amplification; the beam also has special characteristics such as monochromicity, coherence and unidirectionality. Laser sources are divided into three categories namely solid state, gaseous and liquid (dye). Only solid-state and gaseous lasers are considered here and dye lasers generally have low output powers.

1.7.3.1 Solid-state lasers

These include ruby and neodymium yttrium aluminium garnet (Nd:YAG) lasers. These types of lasers have been used in cutting, drilling and marking of materials, military range finders and target designators, a variety of scientific and technology experiments and medical applications.

1.7.3.2 Gas lasers

These include helium-neon and carbon dioxide lasers, which have been used for material processing, marking materials, surgical applications and tactical systems in the military.

1.7.3.3 Laser for decontamination of organisms

Three years after the discovery of the visible light laser, Saks and Roth (162) demonstrated that the ruby laser (wavelength: 694.3 nm) had a significant biocidal effect on *Spirogyra*. To date, the bactericidal effects of laser have been the subject of many studies (**Table 1-9**). The majority of research on laser sterilisation of micoorganisms have concentrated on dental application and the use of laser energy in food industries has been of less concern.

Yanagawa *et al* (220) reported the bactericidal effect of a combination of wavelengths from an argon ion laser on 21 different strains of bacteria. Each organism was exposed to 20, 40 and 60 mW output power, and delivered over 30 min. The results are shown in **Table 1-10.** Gram-positive bacteria were not sensitive to the laser light, and the authors concluded that the rigidity of the cell wall was playing an important part in bacterial resistance. Some Gram-negative bacteria such as *V. parahaemolyticus* also were resistant to laser light. In contrast, others have shown the bactericidal effect of CO_2 laser, with no remarkable difference between Gram types (189).

Ward *et al.* (206) used high-power Nd:YAG laser light (1064 nm wavelength) to inactive bacteria and yeast on agar surfaces. They used different Gram-positive and negative bacteria and yeast and demonstrated that the sensitivity to killing by laser light was not primarily determined by cell size, shape, or clustering, or by Gram-staining characteristics. Watson *et al.* (213) compared bactericidal effects of different lasers and wavelengths on bacteria. Seven laser instruments, delivering radiation at a selection of wavelengths in the range of 0.355 to 118 μm, were investigated for their ability to kill *E. coli* as a lawn on nutrient agar culture plates. A significant ability to kill the bacterium

was observed with the CO_2 (600 W), frequency-tripled Nd:YAG (1 and 0.04 W) and Nd:YAG (200 W) lasers. The different killing efficiencies by the various wavelengths were believed to be partly due to the much higher absorption of radiation at 10.6 μm than at 1.06 μm, by water in the bacterial cells and the surrounding medium (nutrient agar). In another study, the bactericidal effects of laser radiation on *Staphylococcus aureus* were studied with high power Nd:YAG laser radiation between 50 and 300 W. A range of laser pulse repetition frequencies (PRF) from 5 to 30 Hz, with a combination of pulse energies from 2 to 30 J were applied. It was reported that pulse energy, PRF and exposure time were important criteria when considering inactivation of micro organisms by laser radiation (221). A further study was done on *E. coli* in saline suspension. The Nd: YAG laser caused more than 90% loss of viability of the bacterium during exposures that raised the temperature of PBS suspensions of the bacteria to 50°C. In contrast, there was minimal loss of viability after heating the same suspensions to 50°C in a water bath, or in a PCR thermal cycler. The authors concluded that the bactericidal action of Nd:YAG laser light at 50°C was due partly to thermal heating and partly to an additional, as yet undefined, mechanism (207).

Table 1-9. Some experiments on the bactericidal effects of lasers

Laser	Energy	Strains used	Media	Reference	Year
Ruby	3-76 J/cm^{-2}	*Serratia marcescens* *Staph. aureus* *Pseudomonas* *Pneumococcus* *Aspergillus niger*	Solid and in liquid	Klein *et al.* (102)	1965
Helium Neon Ruby Neodymium Glass	25 kW 10 mW 40 mW	*Pseudomonas aeruginosa* *Proteus vulgaris* *Staph. aureus* *Bacillus subtilis*	Liquid and culture media	McGuff and Bell (131)	1966
CO_2	10 W	*Bacillus subtilis* *Clostridium sporogenes* *spores*	Scalpel blades	Adrian and Gross (3)	1979
CO2	10 W	*Bacillus subtilis* *B. stearothermophilus*	Dental root	Hook *et al.* (84)	1980
CO_2	10 W	*Staph. aureus* *E. coli*	Skin seeded	Mullarky *et al.* (138)	1985
CO_2	10 W	*Streptococcus sanguis* *Strep. mutans* *Actinomyces viscosus* *Bacillus cereus* *Staph. aureus* *Pseudomonas aeruginosa*	Glass slides	Zakariasen *et al.* (223)	1986
Nd: YAG	20-120 W	*E. coli* *Staph. aureus* *Pseudomonas aeruginosa*	Wells of microtiter plates	Schultz *et al.* (164)	1986
Argon fluoride excimer	300-330 mJ/cm^{-2}	*Serratia marcescens* *Pseudomonas aeruginosa* *Staph. aureus* *Strep. faecalis* *Haemophilus influenzae* *Candida albicans* *Aspergillus niger*	Agar plates	Keates *et al.* (97)	1988
Xenon chloride excimer	0.7 J/cm^2	*Strep. mutans*	Liquid culture and blood agar	Stabholtz *et al.* (178)	1993
Nd: YAG	400 W	*Staph. aureus*	Nutrient agar	Yeo *et al.* (221)	1998

Sterilization of packaging materials in food industries also has been investigated. A ultraviolet excimer laser, operating at 248 nm, was used to inactivate *Bacillus subtilis* spores deposited on to planar aluminium and polyethylene-coated packaging surface. Kill kinetics were found to be biphasic, with an initial rapid inactivation phase followed by tailing. Although the authors had no definitive evidence, it was thought that spores located within packaging crevices/pores were primarily responsible for the observed tailing. They also found the spores on the unexposed underside of packaging surface. The log count reduction in *B. subtilis* was dependent on spore loading and total UV dose (210). In another study, when polyethylene preformed cartons were exposed to the ultraviolet excimer laser, it was found that the inactivation of the spores and also the distribution of UV was dependent on the type of carton interior coating (209). In a further study it was found that the germination ability of *B. subtilis* spores was altered following UV-excimer laser treatment. The treated spores were recovered in liquid systems (nutrient broth, low acid nutrient broth, whole milk) but not on agar plates supplemented with vegetable extracts or lysozyme (208).

Laser radiation has been investigated for decontamination of surfaces and it was suggested that it could be used for decontamination of containers, and perhaps their contents, on a production line in the food industry (212). The potential of CO_2 laser for decontamination of various foods such as fruits, vegetables and meats, as well as that of solid surfaces, including metals and some plastic has been demonstrated. Carrot and potato inoculated with *E. coli* were exposed to a CO_2 laser beam of 1 kW for pulse duration of 2-10 ms and, after 8 ms, both samples with low inoculum concentration ($1x10^5$ cfu/ml) were completely decontaminated. Also, when higher inoculum concentrations ($1x10^8$ cfu/ml) were used, after 10 ms a 5-log reduction in bacterial count was observed. *Serratia marcescens, S. aureus* and *Pseudomonas aeruginosa* were inoculated onto ham, bacon and fish (whiting and herring) and a low–power laser beam was scanned across the surface of the samples. The potentiality of the laser beam to make a clear area by inactivation of bacteria was observed on bacon, herring skin and ham, but not on the fish flesh. Also, preliminary studies on the nutrient content and lipid oxidation effect of laser-treated ham suggested that exposure had no significant effects (212).

1.7.4 Pulsed electric field (PEF)

Pulses of high-voltage electrical fields are effective in killing bacteria, yeast and moulds in liquid substances (125, 126). This technique is a non-thermal procedure. The antimicrobial effect of a high electric field is due to the ability to cause lethal changes in the cell membrane. If microbes, in a suspension, are exposed to pulses of high external electric field for short time periods (μs) this leads to an increase in the potential difference across the cell membranes. An increase in the membrane potential leads to reduction of membrane thickness. Breakdown of the membrane occurs if the critical breakdown voltage of larger than 1 V is reached through an increase in the external field intensity. In higher electric field strengths, the pore formation on the cell wall becomes irreversible and cell death will happened due to destruction of the membrane functions. This can kill microorganisms in a very short time (136).

Although texture, flavour or colour of the products are not affected, due to the non-thermal nature of the technique, this method can be used only in liquid foods. Also, this method does not inactivate bacterial and fungal spores.

Table 1-10. Effect of argon ion laser on 21 different strains of bacteria.

Organisms (and strain number)	Sensitivity to Laser at energy density		
	72 J/cm^2	144 J/cm^2	216 J/cm^2
Gram-negative aerobic rods			
Pseudomonas aeruginosa 1117	±	+	+
P. fluorescens K-5	±	+	+
P. fluorescens 2101	±	+	+
Acintobacter calcoaceticus	–	–	–
Gram-negative facultatively anaerobic rods			
E. coli 5208	–	±	+
E. coli 3280	–	±	+
E. coli 3284	–	–	±
Shigella sonnei	–	–	–
Salmonella typhimurium	–	–	–
Sal. enteritidis	–	–	±
Proteus mirabilis	–	–	±
Morganella morganii	±	+	+
Serratia marcescens IIa-1	–	–	–
S. marcescens Iva-1	–	–	–
Klebsiella pneumoniae	–	–	–
Vibrio parahaemolyticus 8406-2	–	–	–
V. parahaemolyticus 8406-3	–	–	–
Gram-positive rods			
Bacillus cereus B6-ac	–	–	–
Gram-positive cocci			
Staphylococcus aureus 209P	–	–	–
S. aureus 100	–	–	–
S. aureus 196E	–	–	–

+) If a cleared area with diameter > 6 mm was observed then the bacterial species was regarded as sensitive.

±) For colonies observed growing in the exposed areas, but at a reduced number, the sensitivity was designated questionable.

-) If no visible reduction in the colony formation was observed the bacterial species was regarded as insensitive.

Data from Yanagawa *et al.* (220)

1.7.5 Ozone

Ozone gas (O_3) is a powerful oxidizing agent. In nature, it is continuously produced in the upper atmosphere by the action of solar UV radiation upon oxygen (O_2). Passing oxygen or dry air through a high voltage electric field (by a generator) can form the gas. Ozone has been widely used for disinfection of drinking water and wastewater. The high oxidizing potential and the ability of the gas to diffuse through biological membranes are two of its strong biocidal characteristics. Some studies have shown that the two major pathways for the oxidation reaction of the gas in water are reactions between molecular ozone (direct oxidation) or free radical species formed from the autodecomposition of ozone (indirect oxidation) with some inorganic and organic compounds (53, 81, 82). It is not well understood whether molecular ozone or radical species are responsible for the inactivation of microorganisms. Ozone inactivation kinetics is difficult to measure accurately, because the reactions between the vital components of the microorganisms and ozone are rapid. Also, ozone is rapidly destroyed by autodecomposition and by reactions with other organic components present in solution (87). Komanapalli *et al.* (104) exposed a wild-type and a mutant (DNA repair deficient) strain of *E. coli* to ozone at concentrations of 600 ppm for less than 10 min. Measurements were made of cell viability, glyceraldehyde-3-phosphate dehydrogenase, malate dehydrogenase, lactate dehydrogenase, glutathione disulphide reductase, non-protein sulphydryl and total sulphydryl compounds. The data showed that the most sensitive parameter was glyceraldehyde-3-phosphate dehydrogenase followed by non-protein sulphydryl and total sulphydryl compounds. Also, both the mutant and the wild-type strains were equally sensitive to ozone. It was suggested that the sulphydryl group in the cell membrane is the primary target of ozone attack and the RecA DNA repair system did not appear to play a role in ozone resistance.

The effect of chlorine and ozone on *E. coli* cells resuspended in waste-water has been compared (7). Selected conditions (concentration and contact time) gave a similar decrease (2.5 log) in the bacterial viability. Depending on the disinfectant tested, differences in membrane permeability and cell surface hydrophobicity were observed. Approximately 95.5% of the cells showed altered membrane permeability after ozonation, while no changes in cell surface hydrophobicity were observed. The effect of

chlorine was not linked to changes in membrane permeability. Also, after chlorination, *E. coli* cells showed a tendency to aggregate.

1.7.5.1 Killing effect of ozone on various bacteria

Ozone has been used to kill some bacterial fish pathogens, including *Vibrio anguillarum* in seawater. The results strongly suggested that ozone treatment at more than 1.0 mg of total residual oxidants (TROs) per litre for several minutes was able to disinfect seawater for mariculture efficiently (182). The effects of ozone at 0.25, 0.40 and 1.00 ppm on *L. monocytogenes* in distilled water and phosphate-buffered saline were examined. Differences in sensitivity to ozone were found to exist among the six different strains of *L. monocytogenes*. Greater cell death was found following exposure at lower temperature. Ozone, also at 1.0 ppm concentration completely inactivated all *L. monocytogenes* cells inoculated on cabbage after 5 min (51). Bacteria surviving after ozonation were studied by Lee and Deininger (108). The significant finding was the predominance of Gram-positives (75%) among the surviving bacteria after ozonation. They included *Mycobacterium spp., Bacillus spp., Corynebacterium spp. and Micrococcus spp.*. In another study, Kim and Yousef (101) exposed several types of bacteria to ozone and found that resistance to ozone treatment to be in the order: *E. coli, Pseudomonas, fluorescens, Leuconostoc mesenteroides, and L. monocytogenes.* Also it was found that *S. aureus* was more resistant than *Streptococcus faecalis* and *Candida albicans* when they were exposed to ozone in water (110). The antibacterial activity of ozonated sunflower oil (Olcozon) was studied by Sechi *et al.* (165). Oleozon showed a valuable antimicrobial activity against all microorganisms tested. Results showed that Mycobacteria were more susceptible to Oleozon than the other bacteria tested.

Ozone has been used with other agents for greater effect. For example, a combination of ozone and CO_2 was used and a very effective synergistic effect was observed against a virulent strain of *E. coli* O157. This combination also was effective for bacterial decontamination of black pepper (135). In another study, the bactericidal effect of four different treatments (O_2/O_3, O_2/UV, O_2/O_3/UV and O_2 as the control) was compared. A synergistic effect in reduction of the total aerobic plate count (APC), coliforms, *E. coli*

and *S. typhimurium* was found by using combination of UV and ozone in poultry-processing chiller water. Also, a synergistic reduction in APC bacteria was documented for ozone acting in concert with UV photons as compared with the sum of the effect of O_3 and UV acting in series (42). Combination of pulsed electric field (PEF) and ozone, as two non-thermal processing technologies with potential applications in food industry, was studied by Unal *et al.* (196). They found that exposure of *L. monocytogenes, E. coli* and *Lactobacillus leichmannii* to ozone followed by the PEF treatment had a synergistic bactericidal effect. This synergy was most apparent with a mild dose of ozone against *L. leichmannii.* In another study a strong synergism between ozone and negative air ions (NAI) on bacterial cell death was found, but the degree of this effect varied depending on bacterial species (47).

1.7.5.2 Ozone and the food industry

Oxidizing disinfectants containing chlorine, chloramines and ozone are the final barrier in the Environmental Protection Agency in the USA recommended multibarrier approach to providing pathogen-free water to the consumer. They are the most commonly used disinfectants for drinking water (124). Ozone is a protoplasm oxidant, and its bactericidal action is extremely rapid. The greater oxidation potential and rapid decomposition of ozone are two reasons, which may lead it to be a replacement for chlorine as a common sanitizing agent in the food industry (182). Ozone applications in the food industry are mostly related to decontamination of product surface and water treatment, but it could be used to inactivate contaminant micro-flora on different kinds of foods. Also, ozone is suitable for decontaminating produce, equipment, food-contact surfaces, and processing environments (98). In one study, apples were inoculated with *E. coli* O157:H7 and treated with ozone. Results showed that treatments were more effective when ozone was bubbled during apple washing than by dipping apples in pre-ozonated water (1). This method potentially could be used for other products. Da Silva *et al.* (33) studied sensorial and microbicidal effects of gaseous ozone on different bacteria on agar plates and fresh fish. Five species of fish bacteria, *Pseudomonas putida, S. putrefaciens, Brochothrix thermosphacta, Enterobacter sp.* and *Lacobacillus plantarum*, were inoculated on agar surfaces and exposed to different ozonation times in a gas chamber.

Results showed that low concentrations of ozone (<0.27 x 10^{-3} gl^{-1}) were an effective bactericide of vegetative cells of these organisms. 1 log cfu/cm reduction in viable counts of studied micoorganisms was observed when the bacteria inoculated on the fish skin, were treated with ozone in the laboatory. Also whole fish treated in the laboratory using a commercial ozone generator showed improved scores for sensory analyses compared with the controls. Similar results were obtained when the fish were treated on board ship.

1.8 Scope of the project

Seafood are amongst the most perishable of foodstuffs and novel methods to improve the quality and extend their shelf life would be advantageous. The project set out to investigate the effect of different decontamination technologies, alone or in combination, initially on bacteria in liquid suspension and on agar surfaces and then on different foods. These treatments included laser, UV, microwave, heat and chemical agents. The main target was to achieve minimal processing by investigation of the combination of treatments, in different orders, and to determine the maximum exposure that the produce can take before damage occurs (appearance, sensory evaluation).

Initially, some spoilage organisms and bacteria pathogenic in seafood, as well as *E. coli* as an indicator organism, were tested (in suspension and on agar) against the decontamination technologies (alone and in combination), to identify the most resistant and sensitive bacteria to these treatments. It was considered important to find out the least exposure time using the least energy for killing the bacteria.

The next stage was to identify the killing mechanisms. Investigations included transmission and scanning electron microscopy, measurement of leakage of intracellular constituents (proteins, nucleic acids) and sensitivity of treated cells to lysis by SDS.

The project also focussed on one specific problem in the seafood industry. There are reports of a high incidence of *L. monocytogenes* in processed (smoked) salmon. These studies suggested that although the fish smoking process is effective in killing the bacterium, post-processing contamination occurred (156). There is a high incidence of the bacterium in the smoke-house and salmon slaughterhouse environment. It has been

found that some specific strains of *L. monocytogenes* could survive in the processing plant for several years and continually contaminate the products (117, 156). For these reasons, decontamination of the organism and other spoilage bacteria in the processing area as well as on the products are important and can help to achieve safe products with longer shelf-life.

Chapter 2

MATERIAL and METHODS

CHAPTER 2 MATERIALS AND METHODS

2.1 Bacteria and culture media

2.1.1 Bacteria

***E. coli* (DH5∝ PT7-3):**

This bioluminescent strain was obtained from the culture collection of the Division of Infection and Immunity, University of Glasgow and was used by Ford (52). The bacterium has plasmid pT7-3, which encodes ampicillin resistance and contains the T7 promoter, ϕ 10, and *lux* CDABE genes from *Xenorhabdus luminescens* (188).

***Listeria monocytogenes* (strain R479a):**

Isolated from a cold-smoked salmon processing plant and kindly provided by Dr. L. Gram (200).

***Shewanella putrefaciens* (NCIMB 1732):**

Fish spoilage organism. Obtained from the National Collection of Industrial and Marine Bacteria (NCIMB) Ltd, Aberdeen, UK.

***Pseudomonas fragi* (NCIMB 1353):**

Fish spoilage organism. Obtained from the National Collection of Industrial and Marine Bacteria (NCIMB) Ltd, Aberdeen, UK.

Micrococcus luteus:

Isolated from chilled prawns purchased locally in Glasgow and was identified by the API, NE and 50C, systems. The typical yellow coloured colonies and ease of growth on media like nutrient agar and at 37°C were reasons to select this bacterium.

***Staphylococcus aureus* (strain 24):**

This bacterium is an avian isolate, obtained from the culture collection of the Division of Infection and Immunity, University of Glasgow. The bacterium was originally provided by Dr. McCullagh from a case of proximal femoral degeneration, in a broiler from Northern Ireland.

***Campylobacter jejuni* (strain Col7):**

The bacterium was obtained from the culture collection of the Division of Infection and Immunity, University of Glasgow. Originally it was a clinical isolate from the Public Health Laboratory Service in Colindale.

***Salmonella Typhimurium* (strain 509):**

This bacterium was obtained from the culture collection of the Division of Infection and Immunity, University of Glasgow.

2.1.2 Media

2.1.2.1 Broths

All broths, after preparation, were stored at 4°C until required.

2.1.2.1.1 Nutrient broth

Nutrient broth No. 2 powder (CM 67, Oxoid, 25g) was suspended in one litre of distilled water and boiled until it was dissolved completely. The medium was sterilized at 121°C for 15 min.

2.1.2.1.2 Tryptic soy broth (TSB)

Tryptic soy broth powder (CM 129, Oxoid, 30g) was suspended in one litre of distilled water and boiled until it was dissolved completely. The medium was sterilized at 121°C for 15 min.

2.1.2.1.3 Listeria Enrichment broth (LEB)

Listeria enrichment broth base powder (CM 862, Oxoid, 18g) was suspended in 500 ml of distilled water. The content of one vial of Listeria Selective Enrichment Supplement (SR 141E, Oxoid) was then added to the medium and mixed. The medium was sterilized at 121°C for 15 min.

2.1.2.1.4 Campylobacter enrichment broth (CEB)

Brucella broth powder (Difco, 14g) (50) was suspended in 500 ml of distilled water and boiled until it was dissolved completely. The broth was then sterilised at 121°C for 15 min. Aseptically, 2 ml of distilled water was added to a vial of *Campylobacter* Growth Supplement (SR 084E, Oxoid) and the contents were added to the broth at 45°C.

2.1.2.2 Agars

All agar media were dispensed aseptically into petri dishes under a laminar flow cabinet and then stored at 4°C until required.

2.1.2.2.1 Nutrient agar

Nutrient agar powder (Oxoid, CM3; 28g) was suspended in one litre of distilled water. The mixture was heated to 100°C to dissolve the powder and autoclaved at 121°C for 15 min.

2.1.2.2.2 Nutrient agar plus ampicillin

E. coli (lux) was routinely grown on nutrient medium containing 50 µg/l of ampicillin. Ampicillin (sodium salt, Sigma, 200mg) was dissolved in 20 ml of distilled water as a stock solution. 5 ml from the stock was filter-sterilized (0.2 µm, Minisart®, Sartorious) and added to one litre of sterile medium, cooled to 45°C, to give the required concentration of the antibiotic in the medium.

2.1.2.2.3 Plate count agar

This medium containing casein-peptone glucose yeast extract and is suitable for enumeration of wide range of bacteria. Plate count agar powder (Merck, 22.5g) was suspended in one litre of distilled water. The mixture was heated to 100°C to dissolve the powder and then it was autoclaved at 121°C for 15 min.

2.1.2.2.4 Tryptic soy agar plus yeast extract and glucose (TSA+YG)

Paranjpye *et al.* (147) used this medium to enumerate *L. monocytogenes*. To prepare the medium, Tryptic soy agar powder (Difco, 40g) and yeast extract powder (DUCHEFA, 6g) were suspended in 1 litre of distilled water. The mixture was heated to 100°C to dissolve the powders and autoclaved at 121°C for 15 min. The medium was then cooled to 50°C. 2g of D (+) glucose (Analar®) were dissolved in 5 ml of distilled water and this was then filter-sterilized and added to the medium through a 0.2 µm filter (Minisart®, Sartorious) The medium was mixed and dispensed aseptically into sterile petri dishes.

2.1.2.2.5 Listeria selective agar (Oxford agar)

This agar is a selective and diagnostic medium for detection of *L. monocytogenes*. This medium have alternative supplement containing Amphotericin B as a replacement for cycloheximide. Listeria selective agar base powder (CM856, Oxoid, 27.75g) was suspended in 500 ml of distilled water and boiled until it was dissolved completely. The medium was then sterilized at 121°C for 15 min and cooled down to 50°C. Aseptically,

the content of one vial of Oxford-Listeria-selective-supplement (Merck) was added to the medium and mixed. The medium was dispensed aseptically into sterile petri dishes.

2.1.2.2.6 Listeria monocytogenes blood agar (LMBA)

Listeria monocytogenes Blood agar base powder (LAB M™, 50g) was suspended in one litre of deionised water and boiled until it was dissolved completely. The medium was then sterilized at 121°C for 15 min and cooled down to 47°C. Aseptically, the contents of two vials of X072 (LAB M™) supplement and 50 ml of sterile citrated sheep blood were added to the medium and slowly mixed. The medium was dispensed aseptically into sterile petri dishes

2.1.2.2.7 Campylobacter selective blood agar (CSBA)

Blood agar base No: 2 powder (Oxoid, 40g) was suspended in one litre of distilled water and sterilized. Aseptically, the contents of a vial of Campylobacter Selective Medium (SR098E, Oxoid) and defibrinated sheep blood (5% v/v final) were added to the medium and mixed well. The medium was dispensed aseptically into sterile petri dishes

2.1.2.2.8 Baird-Parker agar (BPA)

Baird-Parker medium powder (CM275, Oxoid, 83g) was suspended in one litre of distilled water and sterilized. Aseptically, 25 ml of the media was pipetted into each plate and allowed to dry.

2.1.2.3 Modified agars

The following selective agars were made for enumeration of bacteria surviving ozone treatment of food samples and also to avoid growth of other bacteria present in samples (chicken skin and smoked salmon).

2.1.2.3.1 Modified Campylobacter Selective Blood agar (MCSBA)

This agar was used for growth of surviving cells of *C. jejuni* after treatment of chicken skin by ozone. It was identical to Campylobacter Selective Blood Agar, described above except that it also contained one vial of Campylobacter growth supplement (SR 084E, Oxoid) per 500 ml of medium.

2.1.2.3.2 Modified Listeria selective agar (MLSA)

This agar was used for growth of surviving cells of *L. monocytogenes* after treatment of smoked salmon by ozone. It was identical to Listeria Selective Agar, described above except that it also contained Listeria Enrichment Broth base powder (CM856, Oxoid, 18 g) and one vial of Listeria Selective Enrichment Supplement (SR 141E, Oxoid) per 500 ml of medium.

2.1.2.3.3 Modified Bismuth Sulphate agar (MBSA)

This agar was used for growth of surviving cells of *S. typhimurium* after treatment of chicken skin by ozone. Mannitol Selenite broth base powder (CM 3998, Oxoid, 19g) was dissolved in 1 litre of distilled water and boiled until it was dissolved completely. After sterilization and cooling down, Bismuth Sulphate agar powder (CM 201, Oxoid, 20g) was dissolved in the broth and heated gently with frequent agitation until the medium just began to boil. It was simmered for 30 sec to dissolve the agar. 25 ml of the medium was pipetted into each plate and allowed to dry.

2.1.2.4 Media and growth conditions for bacteria

Media and incubation conditions used for each strain are shown in **Table 2-1**

2.1.3 Culture methods

2.1.3.1 Preparation of bacterial suspensions (All strains except *C. jejuni)*

A colony from a fresh agar plate culture was inoculated into 50 ml of the appropriate broth (**Table 2-1**). The flask was shaken at 180 rpm for 18-20 h at the appropriate temperature. The culture (15 ml) was then placed in a sterile plastic universal bottle and centrifuged at 4000 rpm for 7 min. The pellet was resuspended in 15ml of normal saline and diluted with sterile physiological saline to compare with the MacFarland standard solutions.

2.1.3.2 Preparation of *C. jejuni* suspensions

1-2 colonies of the bacterium were inoculated into a 100-ml bottle containing 50 ml of Campylobacter Enrichment broth. The bottle was incubated at 37°C in a 200 rpm shaker for 2 h. The suspension was then placed in an anaerobic jar under microaerophilic conditions (80% nitrogen, 15% carbon dioxide and 5% oxygen) for 36 h. The suspension was then centrifuged at 4000 rpm for 7 min and the cells were resuspended into normal saline as described above.

2.1.3.3 Preparation of lawned plates for laser treatment

Nutrient agar (for all strains except *L. monocytogenes*) and Listeria selective agar (for *L. monocytogenes*) plates were made and left to dry for 30 min in a class II laminar air flow cabinet (Flow Laboratories, Germany). Also bacterial suspensions were made as described before and colony counts were made. Then 0.75 ml of the suspensions were pipetted onto the appropriate plates and allowed to flood the surface. The lawned plates were dried for 30 min in a Class III microbiological safety cabinet before use.

Table 2-1. Media and incubation conditions used for each bacterial strain

	Strain	Broth medium	Agar medium	Incubation temperature (°C)
1	*E. coli (lux)*	Nutrient broth + ampicillin	Nutrient agar + ampicillin	37
			TSA	
2	*S. putrefaciens*	Nutrient broth	Nutrient agar	20
3	*P. fragi*	Nutrient broth	Nutrient agar	20
4	*L. monocytogenes*	TSB	TSA	37
			Listeria selective agar	
			Modified Listeria selective agar (MLSA)	
5	*M. luteus*	Nutrient broth	Nutrient agar	37
6	*S. aureus*	TSB	TSA	37
			BPA	
7	*S. typhimurium*	TSB	TSA	37
			Modified Bismuth Sulphate agar (MBSA)	
8	*C. jejuni*	CEB	CSBA	42 (Microaerophilic conditions)
			Modified Campylobacter Selective Blood agar (MCSBA)	

2.1.3.4 Preparation of lawned plates for ozone treatment

Tryptic soy agar (TSA+YG) plates were made as described in section 2.1.2.2.4. Also bacterial suspensions were made in saline for each strain as described before and colony counts were made. 100 µl of the suspension was pipetted onto the surface of TSA+YG agar and spread evenly. Plates were left to dry for 15 min in a Class III microbiological safety cabinet before use. For each strain, 5 plates were made.

2.1.3.5 Colony count method

Generally, 100µl of the bacterial suspension were pipetted into 9.9 ml of sterile normal saline. Ten-fold dilutions were prepared from 10^{-1} to 10^{-7} by pipetting 200 µl into 1.8 ml of saline. From each dilution, 20 µl was pipetted as a drop onto the surface of the appropriate dried agar plates. Each count was set up in duplicate. Plates were incubated overnight and in some cases for up to 1 week at the relevant temperature and any colonies were counted.

2.2 Decontamination systems

2.2.1 UV lamps

UV lamps (3 x 30 W) operating at band C (180-280 nm) were used (**Figure 2-1**). Before using, the lamps were warmed up for 30 min. The energy from the UV lamps was calculated from:

$$\textbf{Energy (J/cm}^2\textbf{) = Power (µW) x Time (s)}$$

The power of the lamps was measured with a UV meter (MaCam®Scotland, UK), which was placed at the target sites. **Table 2-2** shows the power measured at different distances from the lamps.

Through the experiment the energy density was calculated by multiplying the measured power at each distance by the exposure time.

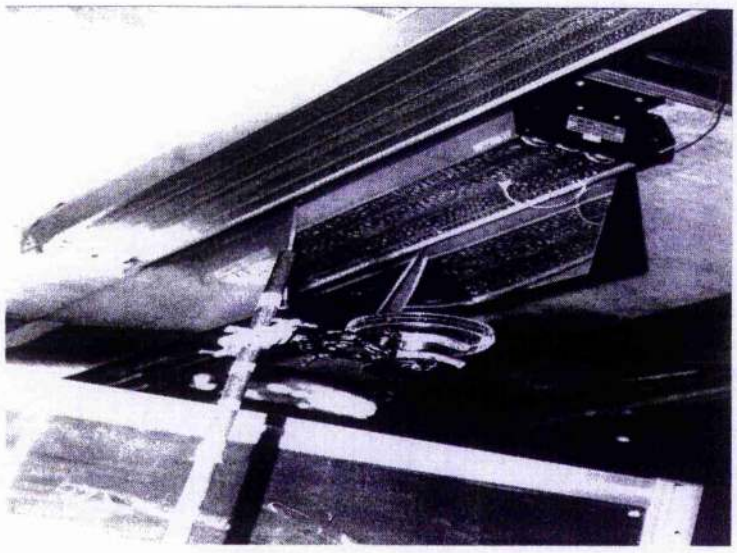


Figure 2-1 Treatment of samples by UV lamps

Table 2-2 Power of UV lamps at different distances

Distance of lamps from the UV meter (cm)	Power of lamps (μWatt)
40	847
50	650
60	540
70	490
80	460

2.2.2 Nd: YAG laser

A pulsed, 400W, Nd:YAG laser (Lumonics, MS830, Rugby, UK) operating at 1064 nm was used, with a fibre optic beam delivery system and collimating focusing lens assembly. The actual laser output power was measured with a power/energy meter (FieldMaster, Coherent, UK). **Table 2-3** shows the calibration of the Nd: YAG laser.

Table 2-3 Calibration of Nd: YAG laser with different parameters

Beam parameter		Set power (W)	Actual power output (W)
Pulse energy (J)	PRF (Hz)		
5	15	75	55
24	5	120	85
8	15	120	92
10	15	150	120
50	5	250	175
24	10	240	177
8	30	240	180
60	5	300	205
20	15	350	217
30	15	450	318

The beam diameter was fixed at 2 cm (when the agar was exposed it was 1.4 cm) on the surface of the suspension. The diameter was measured from burn prints produced on photographic paper (Rypma, 1997). The energy density (ED) of the Nd:YAG laser was calculated from:

ED = Actual power output × Exposure time / Measured beam area (J/cm^2)

The power output (P_o) was obtained from

$$P_o = f \times Pe$$

Where f is the pulse repetition frequency (PRF) and Pe is the pulse energy (J). Throughout the experiments, the laser output pulse energy was set at 20 J, delivered over 8 ms, operating at a PRF of 15 Hz. With these parameters, the calculated ED for 1 sec exposure time was 111.5 (J/cm^2), but the actual measured ED was 69.1(J/cm^2). This

discrepancy was due to losses through the optical system before the laser beam reached the target.

2.2.3 CO_2 laser

A 2000 W gas CO_2 laser (Rofin-Sinar, 1700 SM, Germany) operating at 10600 nm was used. The output beam was delivered to the sample by using a flat mirror. The energy density (ED) of the CO_2 laser is defined as:

Energy density (J/cm^2) = Power (Watts) x Time / Area (J/cm^2)

Power and time were set on the laser control panel and were controlled by a computer. A piece of Perspex® was exposed to different laser powers (W) for 1 sec and the diameter was measured. The beam area was calculated for each power. The results are shown in **Table 2-4**.

Table 2-4. Measured beam diameter and beam area for different power setting of the CO_2 laser

Power (W)	Beam diameter (cm)	Beam area (cm^2)	Power (W)	Beam diameter (cm)	Beam area (cm^2)
10	0.66	0.34	80	1.40	1.54
20	0.67	0.35	90	1.55	1.88
30	0.74	0.43	100	1.60	2.01
40	1.00	0.78	200	1.92	2.89
50	1.10	0.98	300	2.00	3.14
60	1.27	1.27	500	2.06	3.33
70	1.37	1.47	1000	2.07	3.36

2.2.4 Microwave/conventional heating

A domestic microwave oven (800 W, SANYO EMS 153) operated at 2450 MHz was used. Also, a normal laboratory water bath with digital-control of temperature was used for heating the samples for different times.

2.3 Investigation of bacterial treatments

2.3.1 Treatment of bacterial suspensions with UV radiation

A suspension (1.0 ml) of bacterium was pipetted into the wells of multiwell petri dishes (1.7 cm internal diameter) and exposed to the UV light for different times and distances from the lamps. Before exposure, 100µl of the bacterial suspension was removed as the control and viable counts were determined. Immediately after UV exposure, a further sample was taken for viable counting.

2.3.2 Treatment of bacterial suspension with microwave energy

For the controlled application of the microwave treatment, 50 ml of the bacterial suspension was placed into a sterile 200-ml conical flask. The flask was placed into the central cavity of the microwave oven and the samples were exposed for different times. The temperature of the bacterial suspension was obtained by using a digital thermometer before and immediately after treatment. Before and after treatment, 100µl the bacterial suspension was removed for viable counts as a control and to determine the effect of the bacterial reduction due to the microwave.

2.3.3 Treatment of bacterial suspension with Nd: YAG laser radiation

1 ml of bacterial suspension was pipetted into the wells of multiwell petri dishes (1.7 cm internal diameter) and exposed to the laser beam for various times. Before and immediately after treatment, viable cell counts were made as a control and to determine the effect of the laser.

2.3.4 Treatment of bacterial suspensions with ozone

100 ml of bacterial suspension was placed into a sterile 100-ml Duran bottle (suspension was 6 cm deep and 4.5 cm in diameter) and a colony count was made. A generator (Fischer, Badgodesbergi, Germany) was used to create the ozone gas from oxygen. On the ozone generator, the oxygen inlet flow rate was regulated to 100 litres/hour just before the main switch was turned on. Ozone was delivered into the suspension bottle by a rubber pipe with a sterile stainless steel tube (5 mm diameter) at the end. The stainless steel tube was placed, 5.5 cm, into the suspension and the gas was bubbled through to treat the suspension. Exposure times were 2, 5, 10 and 15 minutes. Immediately after treatment, the generator was switched off and the stainless steel tube was removed from the suspension. For safety, a vacuum pump (Nederman®, Sweden) was placed over the bench through the experiment to remove any ozone gas. The treated suspensions were left under vacuum for 1-2 min and then colony counts were made, using the protocol previously described.

2.3.5 Treatment of bacterial suspensions by conventional heating

1.1 ml of bacterial suspension was pipetted into a sterile glass test tube (1 x 10cm) and the tube was placed in a water bath for various times and temperatures. The tube was removed from the water bath and the suspension was left to cool at room temperature. Colony counts were made before and after treatment.

2.3.6 Sequential treatment of bacterial suspensions with UV and laser radiation

1 ml volume of the bacterial suspension was pipetted into the wells of multiwell petri dishes (1.7 cm internal diameter) and exposed to the first treatment. 10 µl from the treated suspension was taken for viable count and the rest of the suspension was exposed to the second treatment. Viable counts were also made after the second treatment.

2.3.7 Sequential treatment of pre-heated (by microwave) bacterial suspensions with UV and laser radiation

50 ml of the bacterial suspension was placed into a sterile 200-ml conical flask and exposed to the microwave radiation as described in section 2.3.2. The flask was then cooled under tap water. Viable cell counts were made and 1 ml from the treated suspension was taken for further treatments by UV and laser as described in section 2.3.6.

2.3.8 Sequential treatment of bacterial suspensions in different orders with laser, UV and microwave radiation

The experiment was designed so as to incorporate all possible orders of treatments with laser, UV and microwave radiation. 1 ml volumes of bacterial suspension were pipetted into the wells of multiwell petri dishes (1.7 cm internal diameter) and exposed to the laser beam or UV radiation as described in sections 2.3.1 and 2.3.3. The treated suspension was then exposed to the laser or UV radiation as second treatment or 500 µl of the treated suspension was pipetted in to a sterile 200-ml conical flask containing 49.5 ml of normal saline (20-fold dilution). The flask was exposed to microwave radiation for 15 sec as described in section 2.3.2 as the second treatment. Due to the nature of the microwave radiation, a small volume of the suspension would not allow a controlled treatment, so a 50 ml volume of suspension was made. Finally, the appropriated third treatment was made. After each treatment, viable counts were made by removing 10 µl volume of suspensions.

2.3.9 Sequential treatment of bacterial suspensions with laser, UV and conventional heating

1.1 ml of the bacterial suspension was exposed to the three treatments in different orders. For treatment with UV or laser, 1.1 ml volume of the suspension were pipetted into wells of the multiwell petri dishes (1.7 cm internal diameter). For treatment with conventional heating, 1.1 ml volume of the suspension was pipetted into a sterile glass test tube (1 x 10cm) and the tube was placed into the water bath at 50 or 55°C for 5 min.

After each treatment, the suspension was pipetted into a sterile test tube and the tube was placed in a 25 °C water bath for 5-6 minutes to cool down under standard conditions. After cooling, 10 µl volumes were taken for viable counts.

2.3.10 Treatment of bacteria on agar plates with ozone

The experiment involved the use of 2 chambers, namely the measurement and the treatment chambers. Prior to treatment, the UV lamps used for ozone measurement were warmed up for 30 min. Both chambers were vented in air before the samples were placed carefully into the treatment chamber. With the four samples in position, Q1 to Q4, (**Figure 2-2**), the lids were immediately sealed. The oxygen inlet flow rate on the ozone generator was regulated to 100 litres/hour, just before the main switch was turned on. Oxygen passing through the corona in the generator rapidly formed ozone gas. A rubber tube fed the ozone into a common pipe connecting both chambers. However, during treatment, the concentration of ozone in the chambers was 6.6 mg/min. A computer recorded the temperature and the drop in UV irradiance as the ozone concentration increased during the ozonation process. After the required treatment time, the ozone generator was switched off. Samples were immediately removed from the chamber and both chambers were vented with a suction fan. Subsequent experiments were conducted identically, varying only the duration of treatment.

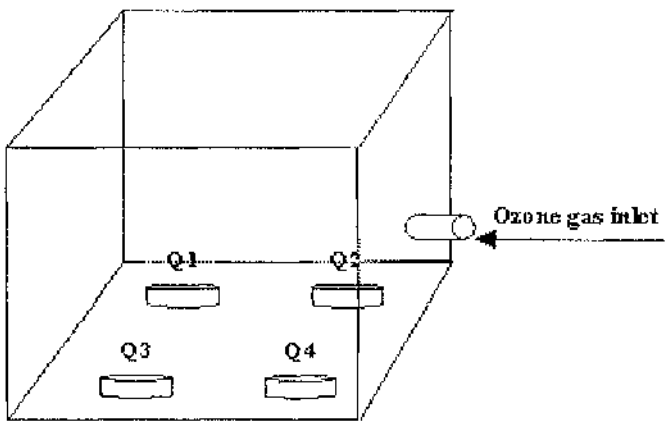


Figure 2-2. Location of lawned agar plates in the treatment chamber during ozonation

2.3.11 Bioluminescence assay

A suspension of *E. coli (lux)* (1.0 ml volume containing 1.3 x 10^9 cfu/ml) was pipetted into the wells of multiwell petri dish and exposed to the UV source (3 x 30 W lamps) for 3, 5, 8, 12 and 20 s at 70 cm or exposed to Nd:YAG laser radiation for 7, 8, 9, 10 and 11 s as described above (sections 2.3.1 and 2.3.3). 50 ml of the suspension was exposed to microwave radiation as described in section 2.3.2 for 10 and 15 s. For exposure to conventional heating, 1 ml of the suspension was pipetted into a test tube and the tube was placed in a water bath for 5, 10, 15 and 20 min at 45, 50, 55°C. After each treatment, 100µl volume of the suspension was removed for viable counting and the rest of the suspension was placed into a disposable measuring cuvette (Polystyrene, Clinicon) for luminometery. All cuvettes were placed into a cold box for transport to the microbiology lab. The time between the end of the treatments and start of the bioluminescence assay was about 2 h. All cuvettes were placed into a luminometer (Model 1251, Bio-Orbit) to measure the light output for 0.5 s, every few minutes over a period of 1 h. Also, one cuvette containing 1ml of saline was used as the control. The mean light output over the measuring time was compared to the cell numbers by viable counts. To make a standard curve, a bacterial suspension was made and then several dilutions, from 10^{-1} to 10^{-7}, were done. Viable cell counts were made and the light output measured for all dilutions.

2.3.12 Bactericidal effect of two laser types on agar plates

By using the Nd: YAG laser, different pulse energies, 4, 8 and 24 Joules, delivered over 8 ms were used and the frequency range was varied between 5 and 60 Hz. The exposure time was adjusted from 8 - 48 seconds. With the CO_2 laser, different frequencies at 100 and 200 W, were used. Each lawned plate was divided into five exposure sites and was mounted on a laboratory jack and positioned beneath the laser beam. Each experiment was repeated in triplicate. After exposure, the plates were incubated overnight at the relevant temperatures and, for more accuracy, each segment was observed under a profile projector to measure the area of clearing. Plates were placed on a projector and the distance between the projector and screen was fixed at 5 times magnification. The average diameters of the clear area where no bacterial growth was

observed were measured for each set of the laser parameters. The areas of bacterial inactivation were calculated and these values were plotted as a function of the energy density at different frequencies.

2.4 Killing mechanisms

2.4.1 Investigation of effect of released cell constituents on protection of bacteria against subsequent treatments

A flask containing 50 ml bacterial suspension was exposed to microwave radiation for 15 sec. The suspension was centrifuged at 4000 rpm for 10 min. 15 ml of the supernate was filtered through a 0.2 μm size sterile filter into a sterile universal bottle. A viable cell count was made to confirm sterilisation of the supernate. In parallel, this procedure was made without any microwave radiation. The optical density (OD) at 260 nm in a quartz cuvette was determined for both supernates against normal saline. 1 ml of fresh bacterial suspension, which was made separately, was pipetted into each solution and viable cell counts were made. 1 ml from each suspension was taken and sequentially exposed to laser and UV radiation as described in section 2.3.6.

2.4.2 Investigation of effect of different cooling methods after microwave treatment on effectiveness of laser treatment

A flask containing of 50 ml of bacterial suspension was exposed to microwave radiation for 15 sec. Three bijou bottles, each containing 1.5 ml of this bacterial suspension were prepared immediately after treatment. Colony counts were made and the first bottle was left at room temperature, the second one was placed on ice for 5 min and the last bottle was placed in a mixture of dry ice and acetone for about 15- 20 sec until frozen. The cooled suspensions were then removed and allowed to reach room temperature. Another colony count was made then 1 ml from each bottle was taken and exposed to laser radiation and a further colony count was made. The same procedure was also done but without any microwave treatment to compare the results.

2.4.3 Measurement of released nucleic acids and protein after different treatments

Bacterial suspensions were exposed to different treatments as described above. The treated suspension was then centrifuged for 8 min at 4000 rpm. The supernate was filtered through the 0.2 µm filter to remove any cells. 100 µl of filtered solution was placed into a quartz cuvette and the OD was obtained against normal saline at 260 and 280 nm.

2.4.4 Effect of different treatments on the sensitivity of *E. coli* to lysis by SDS

As a measure of the level of cell damage (by the various treatments) the sensitivity of cells to lysis by sodium dodecyl sulphate (SDS) was determined. A similar method was used by Woo *et al.* (219) to investigate the damage done to microwave-treated cells. Treated bacterial suspensions were centrifuged for 8 min at 4000 rpm. The supernates were removed and the pellets resuspended in 15 ml of saline. The resulting suspensions were divided into two sterile universal bottles (each containing 7.35 ml) and 150 µl of SDS stock solution (Fisher scientific, 50 mg/ml) was added to the first bottle (0.1% w/v final SDS concentration) and the same amount of normal saline was added to the second bottle. The bottles were placed into 37°C and shaken (180 rpm) for 3 h. The OD at 600 nm against normal saline was obtained for each suspension at 0, 30, 60, 120 and 180 min.

2.4.4.1 Effect of different concentrations of SDS on bacteria

The experiment was done at two temperatures, first at room temperature and the second at 50°C in a water bath (for *E. coli* only). 1 ml of bacterial suspension was added to the SDS solutions to the final SDS concentration (0.01, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5%). Colony counts were made at time 0 and after incubation for 15 and 30 min.

2.4.4.2 Susceptibility of bacteria to killing by SDS after different treatments

Suspensions of the bacterium (1.1 ml volume) were exposed to different treatments and then the suspensions were divided into two sterile universal bottles (each contained 7.35 ml) and 150 µl SDS stock (50 mg/ml for *E. coli* and 5 mg/ml for *L. monocytogenes*) was added to the first bottle (0.1% w/v final SDS concentration for *E. coli* and 0.01% w/v final SDS concentration for *L. monocytogenes*) and the same amount of normal saline was added to the second bottle. The bottles were placed into a 37°C incubator with a shaker (180 rpm) for 1 h. Colony counts were made and the OD at 600 nm against normal saline was obtained for both suspensions at time 0 and after incubation for 30 and 60 min (15 min for *L. monocytogenes*).

2.4.5 Electron microscopy of *E. coli (lux)*

Suspensions of the bacterium (1.1 ml) were exposed to the different treatments as described before. Colony counts were made and scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were done on the samples.

2.4.5.1 SEM processing

All suspensions were centrifuged at 4000 rpm for 3 min and pellets were fixed in 2.5 % w/v glutaraldehyde in phosphate buffer for about 1h. Samples then were rinsed 3 times in 0.1 M phosphate buffer (pH 7.4) for 5 min each and spun down at each change at 4000 rpm for 2-3 min. Samples were stored in buffer at 5°C overnight and post-fixed in 1% w/v osmium tetroxide for 1h. They were washed three times in distilled water for 10 min (spun down at each change at 4000 rpm for 2-3 min). Samples were placed onto Poly-lysine coated cover slips for 30 min and were dehydrated in an alcohol series (30%, 70%, 90%, 100%, and dried 100%) each for 10 min except for absolute alcohol, which was twice for 10 min. They were then critically point dried for 1h 40 min, in Polaron CPD. Cover slips were mounted on double sided copper tape and gold coated then examined using a Philips 500 scanning electron microscope. Images were examined over a range of magnifications (x1600-x12500) and recorded by Image Saver for Windows.

2.4.5.2 TEM processing

All suspensions were centrifuged at 4000 rpm for 3 min and pellets were fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for about 1h, then they were rinsed 3 times in phosphate buffer for 5 min each and spun down at each change. Samples were stored in buffer at 5 °C overnight. Suspensions were post-fixed in 1% w/v osmium tetroxide for 1h. They were washed in distilled water for 10 min, three times (spun down at each change for 2-3 min). Samples were embedded in agarose to produce pellets and then cut into small pieces. Samples were dehydrated in an alcohol series 30% (10 min), 50% (10 min), 70% (overnight at 4°C), 90% (10 min), 100% (2x10 min), and dried 100% (10 min). They were then put in propylene oxide for three changes, each of 5 min. The samples then were put into tubes containing 1:1 propylene oxide:araldite resin. They were rotated a few hours with the cap on and then left over night with cap off to allow propylene oxide to evaporate. The samples were transferred to fresh Araldite resin the next day. The samples were embedded in resin and left in an oven at 60°C to be polymerised for 48h. 70-80 nm sections were made by using a Reictert ultera microtome and mounted on 300 mesh formvar-coated copper grids. The sections were contrast-stained by 2% w/v uranyl acetate in methanol for 5 min and lead citrate for 5 min and were examined by Zeiss 902 TEM.

2.5 Decontamination of selected foodstuffs

2.5.1 Total bacterial count of smoked salmon

Packs of smoked salmon were purchased from various local shops and opened aseptically. 1g of smoked salmon was placed in 9 ml of peptone saline (10g Bacto-peptone, Difco) in 1 litre of normal saline and sterilized). In a class III safety cabinet, this mixture was homogenised by a mixer-emulsifier (Silverson Machines Ltd, London) and dilutions from 10^{-1} to 10^{-3} were made. Nine plates containing plate count agar in three groups were prepared and 100 µl of each dilution was spread onto the three plates and

allowed to dry. The first group was placed at 18°C, the second at 30°C and the last one at 37°C for 48 h and any colony forming units were counted.

2.5.2 Isolation and enumeration of *L. monocytogenes* in smoked salmon

Smoked salmon (1g) under aseptic condition was placed in 9 ml of Listeria Enrichment Broth (LEB). In a class III safety cabinet, this mixture was homogenised and 100 µl of the mixture was spread on Oxford agar and LMBA. The plates were incubated at 37°C for 48 h to detect any colonies directly from the sample. The rest of the mixture was incubated at 30°C for 48 h on a shaker (150 rpm) and, after 24 and 48 h, 100 µl of the mixture was plated on Oxford agar and LMBA. The plates were incubated at 37°C for 48 h to find any *Listeria*. Gram staining, oxidase and catalase tests were done on the suspected colonies and identity confirmed by API®Listeria kit (Bio Merieux, France).

2.5.3 Decontamination of smoked salmon by sequential treatment of UV, laser and conventional heating

Packs of smoked salmon were purchased from various local shops and opened aseptically. Total counts and Listeria counts (or *E. coli* counts) were made as described above. Then the salmon slices were divided into several 1.5 cm diameter pieces (about 0.4 g weight) with a No 8 cork borer. Each piece was placed in a sterile petri dish. A suspension of bacteria was made and 10 µl of the suspension was placed on the top of the sample and allowed to dry for about 15 min. Three samples were taken as controls and the rest of the samples (in groups of 3) were exposed to the various treatments. Each sample was put into 9.6 ml of saline and homogenized. The mixture was diluted and colony counts were done on the appropriate agar. Plates were incubated at 37°C for 48 h and colonies were counted.

2.5.4 Decontamination of *L. monocytogenes* on smoked salmon by ozone

Modified Listeria selective agar (MLSA) was used to isolate *L. monocytogenes* form the smoked salmon samples.

Smoked salmon slices were divided into several pieces with a No 7 cork borer (about 1cm in diameter) and weighed. Each piece was placed into a sterile petri dish. A suspension of *L. monocytogenes* was made and 10 µl of the suspension was placed on the top of the sample and allowed to dry for about 15 min. A total of 11 smoked salmon samples were prepared, three were used as controls, 4 samples were treated for 10 minutes with ozone and the rest of the sample were treated for 15 minutes. Ozonation were carried out in same way as described in section 2.3.10. After treatment, the samples were transferred into universal bottles filled with 9 ml of Listeria enrichment broth. The samples were homogenised before plating 100 µl onto MLSA. Colony counts were obtained after incubation for 48 h at 37°C.

2.5.5 Decontamination of selected bacteria on chicken skin by ozone

Chicken skin was aseptically removed from their carcasses and divided into several pieces with a No 8 cork borer. Each piece was weighed and placed on a sterile petri dish. Similar to the last section, 11 samples were prepared for each bacterium. 10µl of the bacterial suspension was placed on each sample and then the samples were treated with ozone for 10 and 15 minutes. After treatment, each sample was transferred into a sterile glass universal bottle containing 9 ml of peptone saline and shaken at 200 rpm for 15 minute before plating the 100µl onto the appropriate modified selective agar. These plates were incubated under the appropriate conditions for 48h and any colonies counted.

2.5.6 Statistical analysis of data

Each experiment was repeated 3 times for statistical reliability. By using Microsoft's Excel programme, the mean of the results, log reductions in viable counts and standard deviations were calculated for each set of experiments and also the results were plotted. A 1-log reduction in viability is equivalent to 90% reduction in viable count i.e. 90%

killing, a 2-log reduction is equivalent to 99% reduction in viable count, 3-log reduction is equivalent to 99.9% reduction in viable count, 4-log reduction is equivalent to 99.99% reduction in viable count etc.

Results from the statistical software program Graph Pad Instant were determined by using the statistical tests: one-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparison tests, it was assumed that if $P<0.05$ the test was significant.

Chapter 3

RESULTS

CHAPTER 3 RESULTS

3.1 The killing effect of UV, microwave and Nd:YAG laser radiation on selected bacteria in saline suspension

A series of treatments was made to identify the most resistant and sensitive bacteria to each treatment (alone or sequentially) and to determine the least exposure time using the minimum energy for killing the bacteria. *Shewanella putrefaciens, Pseudomonas fragi* and *Micrococcus luteus* were chosen as examples of Gram-negative and Gram-positive spoilage bacteria that are important in seafood. *Listeria monocytogenes* was chosen as a pathogenic bacterium that can potentially be transferred to the consumer via seafood and has frequently been isolated in smoked salmon products. *E. coli is* commonly used as an indicator organism in the food industry and a bioluminescent construct, *E. coli (lux),* was selected in order to assess the feasibility of using light output measurements to monitor bacterial killing.

3.1.1 Treatment of bacterial suspension with UV radiation

Exposure times for Gram-negative bacteria were 3, 5 and 8 sec at 50 (650 μWatt/cm^2/s), 60 (540 μWatt/cm^2/s) and 70 (490 μWatt/cm^2/s) cm distance from the lamps. Exposure times for *M. luteus* were varied between 10, 15 and 20 sec at 40 (847 μWatt/cm^2/s), 50 and 60 cm from the lamps. Colony counts were made before and immediately after treatments. All experiments were repeated 3 times. **Tables 3-1, 3-2, 3-3** and **3-4** show the average number of surviving bacteria and the log reduction in viable counts as a function of distance from the UV lamps and different exposure times. **Figure 3-1** shows a comparison of the killing effect of UV radiation on the selected bacteria. The limit of detection of viable count is also shown.

It can be see that UV was effective in killing these bacteria but the Gram-negative strains were more sensitive than the Gram-positive bacterium. Among the Gram-negative bacteria tested, *E. coli (lux)* was the most sensitive, then *P. fragi* then *S. putrefaciens*. A

high standard deviation was observed at low exposure times. This may have been due to the necessity to switch off the UV lamps for a few seconds in order for the operator to move in and out of the lamp guard area between treatments. This could have affected the power of the UV lamps and account for the variable killing effect with short exposure times. 2700 μW s/cm^2 produced more than a 6-log reduction in viable counts on *E. coli (lux)*. The same energy density produced a 2.55 and 2.92 log reduction respectively in the viability of *P. fragi* and *S. putrefaciens*, but 11000 μW s/cm^2, was necessary for a 2-log reduction in the viability of the *M. luteus*.

Table 3-1. Killing effect of UV radiation on *S. putrefaciens* in saline suspension

Time (sec)	Distance (cm)	Energy density (μWatt s/cm^2)	Mean* of survival of bacteria after treatment (cfu/ml)	Log reduction (cfu/ml)	Log reduction STDEV**
Control	-	**0**	**4.5×10^8**	-	**0.09**
3	70	1470	2.8×10^7	1.20	0.34
3	60	1620	4.0×10^7	1.05	0.58
3	50	1950	6.2×10^6	1.86	0.50
5	70	2450	9.0×10^6	1.70	0.44
5	60	2700	1.3×10^6	2.55	0.56
5	50	3250	4.1×10^5	3.04	0.80
8	70	3920	6.6×10^5	1.83	0.31
8	60	4320	2.3×10^4	4.28	0.28
8	50	5200	2.0×10^4	4.34	0.25

*** Mean of 3 observations**

****STDEV: Standard deviation**

Table 3-2. Killing effect of UV radiation on *P. fragi* in saline suspension

Time (sec)	Distance (cm)	Energy density (μWatt s/cm^2)	Mean of survival of bacteria after treatment (cfu/ml)	Log reduction (cfu/ml)	Log reduction STDEV
Control	-	**0**	**2.6×10^8**	-	**0.26**
3	70	1470	1.7×10^7	1.38	0.33
3	60	1620	1.2×10^6	2.34	0.62
3	50	1950	1.3×10^6	2.31	0.33
5	70	2450	4.4×10^6	1.77	0.25
5	60	2700	3.1×10^5	2.92	0.56
5	50	3250	1.5×10^4	4.25	0.54
8	70	3920	9.5×10^2	5.43	0.04
8	60	4320	9.1×10^2	5.45	0.05
8	50	5200	<50	>6.41	

Table 3-3. Killing effect of UV radiation on *E. coli (lux)* in saline suspension

Time (sec)	Distance (cm)	Energy density (μWatt s/cm^2)	Mean of survival of bacteria after treatment (cfu/ml)	Log reduction (cfu/ml)	Log reduction STDEV
Control	**-**	**0**	**3.6×10^8**	**-**	**0.06**
3	70	1470	1.3×10^7	1.43	0.32
3	60	1620	5.1×10^6	1.85	0.17
3	50	1950	2.2×10^5	3.20	0.40
5	70	2450	5.1×10^6	1.84	0.17
5	60	2700	<50	>6.85	
5	50	3250	<50	>6.85	
8	70	3920	<50	>6.85	
8	60	4320	<50	>6.85	
8	50	5200	<50	>6.85	

Table 3-4. Killing effect of UV radiation on *M. luteus* in saline suspension

Time (sc)	Distance (c)	Energy density (μWatt s/cm^2)	Mean of survival of bacteria after treatment (cfu/ml)	Log reduction (cfu/ml)	STDEV
Control	**-**	**0**	**1.2×10^8**	**-**	**0.09**
10	60	5400	4.5×10^7	0.44	0.20
10	50	6500	1.2×10^7	1.02	0.09
15	60	8100	3.5×10^6	1.56	0.42
10	40	8470	1.6×10^7	0.91	0.33
15	50	9750	2.0×10^6	1.80	0.11
20	60	10800	2.2×10^6	1.76	0.31
15	40	12705	2.3×10^5	2.74	0.34
20	50	13000	4.4×10^5	2.46	0.50
20	40	16940	3.2×10^4	3.59	0.26

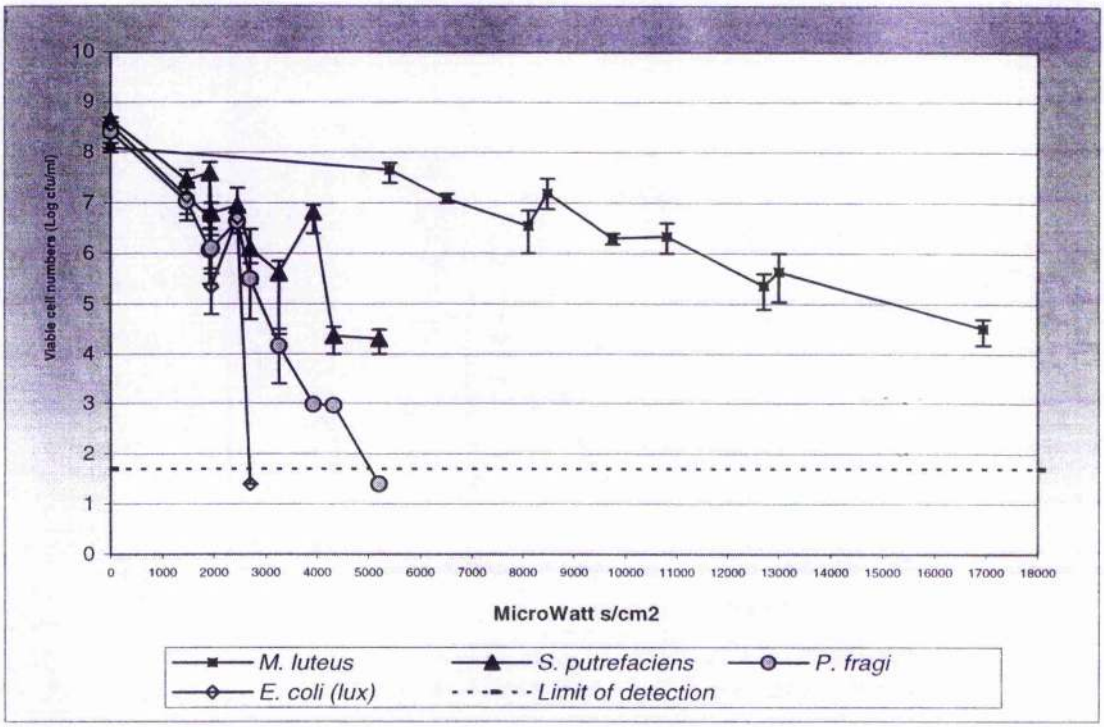


Figure 3-1. Comparison of the killing effect of UV radiation on selected bacteria in saline suspension

3.1.2 Treatment of bacterial suspensions with microwave energy

For the controlled application of microwave energy, a large volume (50 ml) of bacterial suspension was used throughout these experiments. All experiments were repeated 3 times. The results are shown in **Tables 3-5, 3-6, 3-7 and 3-8 and in Figures 3-2 and 3-3.** There was little reduction in viable counts after treatment of *S. putrefaciens* and *P. fragi* for up to 15 min but, after that, survival decreased sharply to less than 50 cfu/ml (the limit of detection) for both bacteria. This happened when the temperature was raised to 71°C or more, during 20 s of treatment. There was some temperature variation between 68.4 to 76.7 °C, when suspensions of *E. coli* and *M. luteus* were treated for 21 s. Some colonies were detected when the fluid temperature was < 71°C but, as with the previous strains, viable counts decreased sharply when the temperature was raised to > 71°C.

From these data it is difficult to say which bacteria were most sensitive to microwave treatment, but generally it seemed that *S. putrefaciens* and *P. fragi* were slightly more sensitive to treatment than *E. coli* and *M. luteus*. Due to the nature of microwave radiation, control of accretion of temperature through the exposure time was very difficult and the size, shape and place of the container during treatment were critically important. With the procedure used, a temperature between 70-71°C was the critical point for killing bacteria by microwave energy.

Table 3-5. Killing effect of microwave radiation on *S. putrefaciens* in saline suspension

Exposure time (sec)	Temperature range (°C)	Mean of survival of bacteria after treatment (cfu/ml)	Log reduction (cfu/ml)	Log reduction STDEV
Control	**21.5-22.2**	**1.5×10^8**	-	**0.39**
5	26.5-29.1	9.4×10^7	0.21	0.31
10	42-43.8	5.0×10^7	0.47	0.20
15	52.5-53	4.1×10^7	0.56	0.50
20	71-71.5	< 50	>6.78	

Table 3-6. Killing effect of microwave radiation on *P. fragi* in saline suspension

Exposure time (sec)	Temperature range (°C)	Mean of survival of bacteria after treatment (cfu/ml)	Log reduction (cfu/ml)	Log reduction STDEV
Control	**21.5-22**	**1.8×10^8**	-	0.22
5	27-28.6	1.1×10^8	0.26	0.16
10	41.5-44.5	1.0×10^8	0.26	0.23
15	50.5-56.5	6.8×10^7	0.43	0.63
20	71.6-73	< 50	>6.86	

Table 3-7. Killing effect of microwave radiation on *E. coli (lux)* in saline suspension

Exposure time (sec)	Temperature range (°C)	Mean of survival of bacteria after treatment (cfu/ml)	Log reduction (cfu/ml)	Log reduction STDEV
Control	**21.7-23.5**	**3.1×10^8**	-	**0.09**
15	49.6-51.5	1.6×10^8	0.27	0.59
18	57.9-68.3	1.0×10^8	0.48	1.11
21	68.4-76.7	6.7×10^6*	1.67	3.14
24	78.4-87	<50	>7.09	

***Value when temperature of suspension reached 68.4°C. No cfu was detected when the temperature reached to 76.7°C and 300 cfu/ml were detected at 70°C**

Table 3-8. Killing effect of microwave radiation on *M. luteus* in saline suspension

Exposure time (sec)	Temperature range (°C)	Mean of survival of bacteria after treatment (cfu/ml)	Log reduction (cfu/ml)	Log reduction STDEV**
Control	**20.8-21.8**	**4.6×10^8**	-	**0.09**
12	48.7-51.5	3.0×10^7	0.26	0.59
15	52.3-58.8	2.7×10^7	0.31	1.11
18	60.7-68.1	1.1×10^7	0.70	3.14
21	70.3-76.4	8.4×10^3*	3.82	0
24	81.2-86.6	<50	>6.34	

***Bacteria were detected only when temperature was 70.3°C**

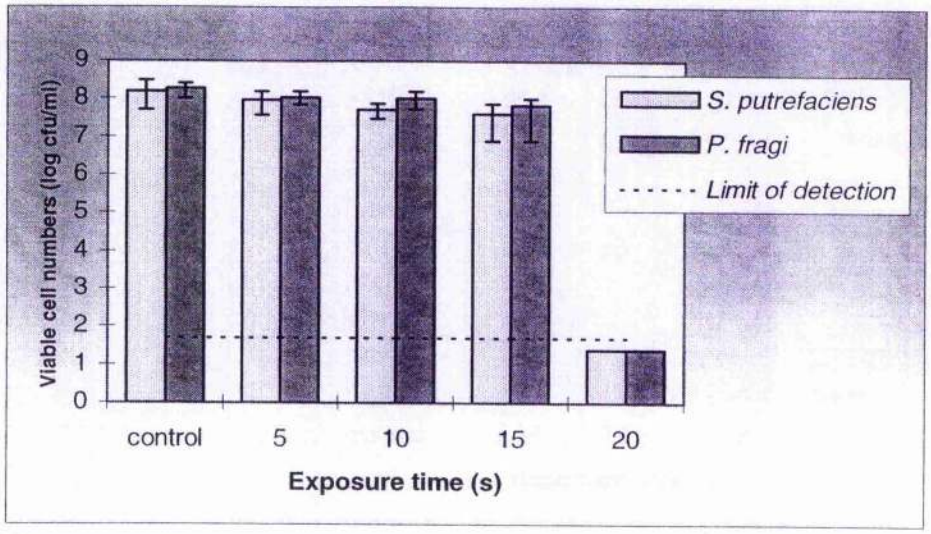


Figure 3-2. Comparison of killing effect of microwave energy on selected bacteria in saline suspension (1)

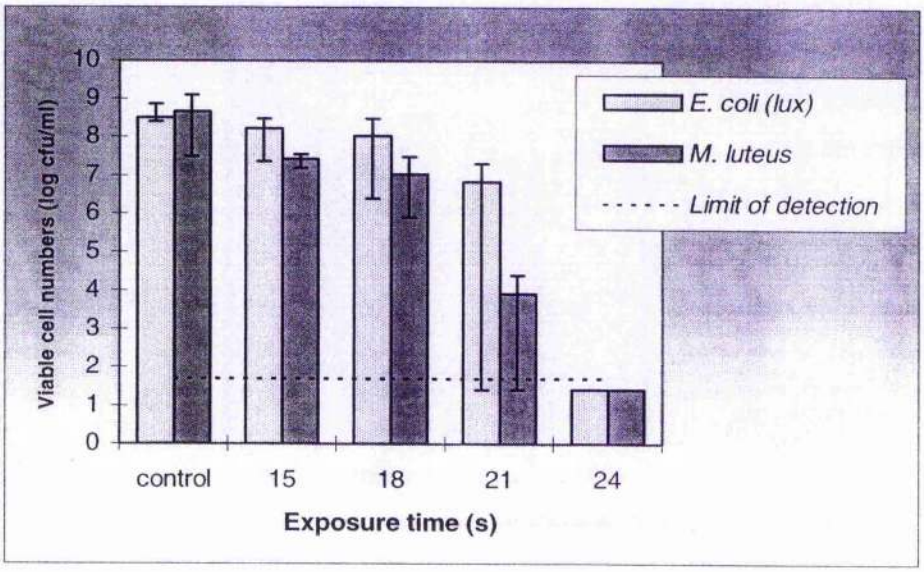


Figure 3-3. Comparison of killing effect of microwave energy on selected bacteria in saline suspension (2)

3.1.3 Treatment of bacterial suspensions with Nd:YAG laser radiation

Tables 3-9, 3-10, 3-11 and 3-12 show the killing effect of the laser on each bacterium. **Figure 3-4** shows a comparison of the killing effect of the laser on these bacteria. Except for *S. putrefaciens,* energy less than 550 J/cm^2 did not produce a significant reduction in viable counts. However, a log reduction of more than 6.89 was apparent from the viable counts of *S. putrefaciens* with this energy density. Survival of *P. fragi*, *E. coli* and *M. luteus* was below the limit of detection (50 cfu/ml, >6 log reduction) after 691, 760.1 and 829.2 J/cm^2 of laser energy, respectively. Thus, laser energy from the Nd:YAG laser was effective in killing bacteria in suspension in a short time (few sec). No large difference was seen in the laser energy density required for killing Gram-positive or Gram-negative bacteria, although *M. luteus* proved to be the most resistant. *S. putrefaciens* and *P. fragi* were more sensitive to the laser treatment than *E. coli.*

Table 3-9. Killing effect of Nd:YAG laser radiation on *S. putrefaciens* in saline suspension

Time (sec)	Energy density (J/cm^2)	Mean of survival of bacteria after treatment (cfu/ml)	Log reduction (cfu/ml)	Log reduction STDEV
Control	**0**	**3.9×10^8**	**0**	**0.04**
3	207.3	2.9×10^8	0.13	0.15
4	276.4	2.1×10^8	0.27	0.08
5	345.5	1.4×10^8	0.45	0.65
6	414.6	1.1×10^8	0.56	0.14
7	483.7	2.5×10^7	1.19	0.30
8	552.8	<50	>6.89	-

Table 3-10. Killing effect of Nd:YAG laser radiation on *P. fragi* in saline suspension

Time (sec)	Energy density (J/cm^2)	Mean of survival of bacteria after treatment (cfu/ml)	Log reduction (cfu/ml)	Log reduction STDEV
Control	**0**	**4.7×10^8**	**0**	**0.06**
5	345.5	4.4×10^8	0.03	0.08
6	414.6	4.0×10^8	0.07	0.09
7	483.7	2.6×10^8	0.25	0.26
8	552.8	2.7×10^8	0.24	0.05
9	621.9	7.9×10^6	1.77	0.61
10	691.0	<50	>6.97	-

Table 3-11. Killing effect of Nd:YAG laser radiation on *E. coli* in saline suspension

Time (sec)	Energy density (J/cm^2)	Mean of survival of bacteria after treatment (cfu/ml)	Log reduction (cfu/ml)	Log reduction STDEV
Control	**0**	**3.9×10^8**	**0**	**0.11**
7	483.7	3.8×10^8	0.01	0.11
8	552.8	3.2×10^8	0.09	0.18
9	621.9	1.3×10^8	0.46	0.63
10	691.0	1.0×10^6	2.58	0.62
11	760.1	<50	>6.89	-

Table 3-12 Killing effect of Nd:YAG laser radiation on *M. luteus* in saline suspension

Time (sec)	Energy density (J/cm^2)	Mean of survival of bacteria after treatment (cfu/ml)	Log reduction (cfu/ml)	Log reduction STDEV
Control	**0**	**1.7×10^8**	**0**	**0.16**
8	552.8	1.5×10^8	0.06	0.15
9	621.9	4.5×10^7	0.59	0.14
10	691.0	2.2×10^7	0.90	0.15
11	760.1	1.1×10^6	2.19	0.87
12	829.2	<50	>6.54	-

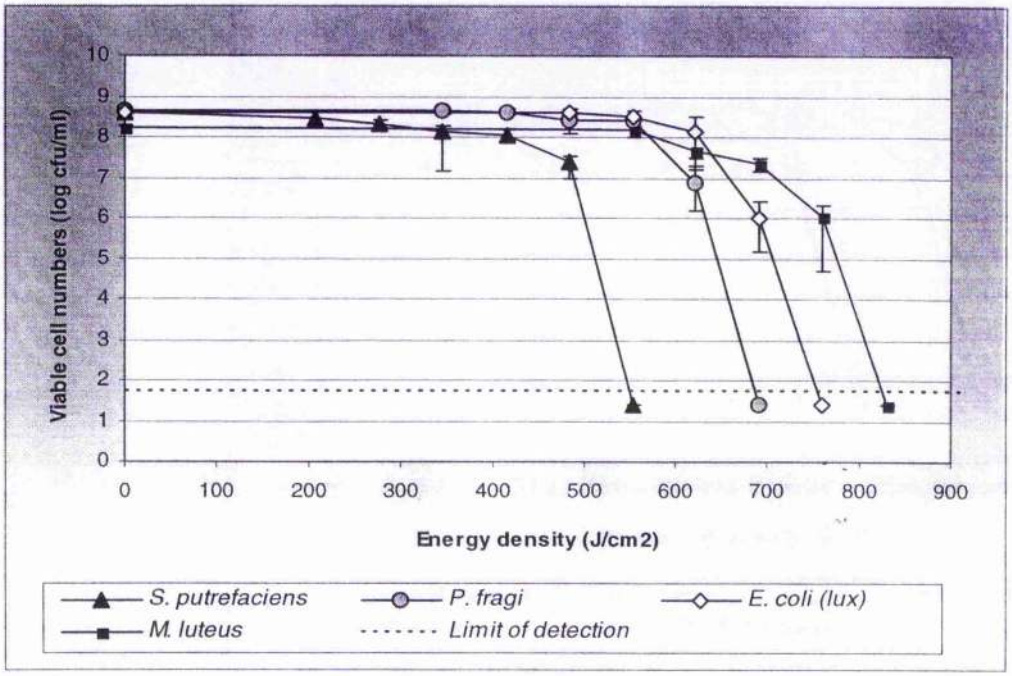


Figure 3-4. Comparison of the killing effect of Nd:YAG laser on selected bacteria in saline suspension

3.2 Treatment of bacterial suspensions with combination of UV and laser radiation

The killing effect of combined UV and laser treatments, sequentially, was investigated. The lowest energy densities for each technique that produced a smallest significant reduction in viable count, were chosen for the combination experiments. The parameters used for each bacterium are listed below:

Bacteria	*S. putrefaciens*	*P. fragi*	*E. coli*	*M. luteus*
Laser parameters	6 sec (414.6 J/ cm^2) 7 sec (483.7 J/ cm^2)	8 sec (552.8 J/ cm^2) 9 sec (621.9 J/ cm^2)	9 sec (621.9 J/ cm^2) 10 sec (691 J/ cm^2)	10 sec (691 J/ cm^2) 11 sec (760.1 J/ cm^2)
UV parameters	3 sec/ 60 cm (1620 µwatt s/cm^2) 5 sec/ 60 cm (2700 µwatt s/cm^2)	3 sec/ 70 cm (1470 µwatt s/cm^2) 5 sec/ 70 cm (2450 µwatt s/cm^2)	3 sec/ 70 cm (1470 µwatt s/cm^2) 5 sec/ 70 cm (2450 µwatt s/cm^2)	10 sec/ 60 cm (5400 µwatt s/cm^2) 15 sec/ 60 cm (8100 µwatt s/cm^2)

The survival and mean of the log reduction in viable counts for the different bacteria by each set of parameters in different sequences on the bacteria is shown in **Tables 3-13, 3-15, 3-17 and 3-19**, also the mean of the log reduction by each treatment alone and the sum of the log reduction by the individual treatments shown in **Tables 3-14, 3-16, 3-18 and 3-20.** The results show that the response of each strain to the combined treatments was slightly different.

S. putrefaciens: In comparison to the sum of the log reductions by individual treatments, a slight synergistic effect (about 0.5 log greater reduction in viable counts) was observed. This was only seen for the combination of 3 sec UV and 6 sec laser exposure. No greater reduction in viable count was observed for other energies compared to sum of log reduction by two treatments separately. Generally, however for this bacterium, the order: UV then laser showed a better killing effect than laser then UV.

P. fragi: In contrast to the previous strain, killing by the combination of two treatments with UV then laser was not significant compared to the sum of the log

reductions of the individual treatments. However, about a 1 log greater reduction in viable counts was monitored with laser then UV.

E. coli (lux): Similar to *P. fragi*, an increase of about 0.3-0.5 log reduction in viable counts was monitored with 10 sec laser followed by 5 sec UV radiation (the highest parameters). The killing effect of lower energies was similar to the sum of killing from individual treatments.

M. luteus: The results showed a significant synergistic effect by combined treatments on the only Gram-positive bacterium tested. A reduction in the viable counts was apparent with all sequences and levels of energies. About 0.55, 0.57, 0.89 and 1.2 greater log reductions in viability were apparent by combination of 10 sec laser/10 sec UV, 10 sec laser/15 sec UV, 11 sec laser/10 sec UV and 11 sec laser/15sec UV, respectively, compared to the sum of each treatment alone. Also the killing effect with laser then UV was greater than UV then laser.

Comparisons of the killing effect of different sequential treatments on the bacteria tested are shown in **Figures 3-5, 3-6, 3-7 and 3-8.** There was a slight synergistic effect on bacterial killing when two treatments were combined in comparison to the sums of the killing effect of each treatment alone. Despite some differences, it seemed that laser followed by UV gave better killing than UV followed by laser. The synergistic effect was more noticeable with *M. luteus*, a Gram-positive bacterium, than with the Gram-negative bacteria. Interestingly the synergistic effect was increased when higher energies were used.

Table 3-13. Killing effect of combination of UV and laser on *S. putrefaciens* in saline suspension

First treatment	Mean of survival of bacteria (cfu/ml)	Log reduction	STDEV	Second treatment	Mean of survival of bacteria (cfu/ml)	Total log reduction	STDEV
Control	6.5×10^8	0	0.12	-			
6 sec laser	3.5×10^8	0.26	0.06	3 sec UV	2.5×10^7	1.42	0.33
6 sec laser	3.3×10^8	0.29	0.04	5 sec UV	2.2×10^6	2.46	0.30
7 sec laser	1.0×10^8	0.80	0.45	3 sec UV	3.0×10^7	1.34	0.60
7 sec laser	8.8×10^7	0.87	0.14	5 sec UV	1.9×10^6	2.53	0.63
3 sec UV	3.7×10^7	1.25	0.22	6 sec laser	5.5×10^6	2.07	0.17
5 sec UV	4.3×10^6	2.18	0.29	6 sec laser	1.8×10^6	2.55	0.13
3 sec UV	5.2×10^7	1.10	0.14	7 sec laser	4.2×10^6	2.19	0.29
5 sec UV	4.5×10^6	2.16	0.27	7 sec laser	8.7×10^5	2.88	0.05

Table 3-14. Killing effect of separate treatments on *S. putrefaciens*

Treatment	Mean of log reduction (cfu/ml)	Treatments	Sum of mean of log reduction (cfu/ml)
3 sec UV alone	1.17	6 sec laser and 3 sec UV separately	1.44
5 sec UV alone	2.17	6 sec laser and 5 sec UV separately	2.44
6 sec laser alone	0.27	7 sec laser and 3 sec UV separately	2.00
7 sec laser alone	0.83	7 sec laser and 5 sec UV separately	3.00

Table 3-15. Killing effect of combination of UV and laser on *P. fragi* in saline suspension

First treatment	Mean of survival of bacteria (cfu/ml)	Log reduction	STDEV	Second treatment	Mean of survival of bacteria (cfu/ml)	Total log reduction	STDEV
Control	4.5×10^8	0	0.36				
8 sec laser	2.2×10^8	0.30	0.28	3 sec UV	5.9×10^6	1.88	0.94
8 sec laser	2.7×10^8	0.22	0.21	5 sec UV	3.6×10^5	3.09	0.48
9 sec laser	4.7×10^7	0.98	0.64	3 sec UV	3.0×10^6	2.17	1.30
9 sec laser	2.6×10^7	1.23	0.67	5 sec UV	8.7×10^3	4.71	0.40
3 sec UV	7.5×10^7	0.87	0.31	8 sec laser	5.0×10^7	0.95	0.67
5 sec UV	1.1×10^7	1.63	0.61	8 sec laser	3.1×10^6	2.16	0.78
3 sec UV	6.8×10^7	0.82	0.44	9 sec laser	2.3×10^6	2.29	0.78
5 sec UV	9.1×10^6	1.69	0.34	9 sec laser	3.5×10^5	3.13	0.75

Table 3-16. Killing effect of separate treatments on *P. fragi*

Treatment	Mean of log reduction (cfu/ml)	Treatments	Sun of mean of log reduction (cfu/ml)
3 sec UV alone	0.84	8 sec laser and 3 sec UV separately	1.10
5 sec UV alone	1.66	8 sec laser and 5 sec UV separately	1.92
8 sec laser alone	0.26	9 sec laser and 3 sec UV separately	1.99
9 sec laser alone	1.15	9 sec laser and 5 sec UV separately	2.81

Table 3-17. Killing effect of combination of UV and laser on *E. coli (lux)* in saline suspension

First treatment	Mean of survival of bacteria (cfu/ml)	Log reduction	STDEV	Second treatment	Mean of survival of bacteria (cfu/ml)	Total log reduction	STDEV
Control	8.3×10^8	0	0.12	-			
9 sec laser	4.2×10^8	0.29	0.15	3 sec UV	4.7×10^7	1.25	0.36
9 sec laser	3.6×10^8	0.36	0.20	5 sec UV	8.0×10^6	2.02	0.60
10 sec laser	5.7×10^7	1.17	0.33	3 sec UV	1.7×10^6	2.70	0.07
10 sec laser	3.3×10^7	1.40	0.40	5 sec UV	2.9×10^5	3.46	0.31
3 sec UV	6.7×10^7	1.10	0.17	9 sec laser	4.5×10^7	1.27	0.14
5 sec UV	2.3×10^7	1.55	0.06	9 sec laser	1.6×10^7	1.70	0.27
3 sec UV	8.5×10^7	0.99	0.22	10 sec laser	2.2×10^6	2.58	0.13
5 sec UV	2.7×10^7	1.49	0.35	10 sec laser	2.3×10^6	2.55	0.16

Table 3-18. Killing effect of separate treatments on *E. coli (lux)*

Treatment	Mean of log reduction (cfu/ml)	Treatments	Sum of mean of log reduction (cfu/ml)
3 sec UV alone	0.32	9 sec laser and 3 sec UV separately	1.32
5 sec UV alone	1.28	9 sec laser and 5 sec UV separately	1.84
9 sec laser alone	1.00	10 sec laser and 3 sec UV separately	2.28
10 sec laser alone	1.52	10 sec laser and 5 sec UV separately	2.80

Table 3-19. Killing effect of combination of UV and laser on *M. luteus* in saline suspension

First treatment	Mean of survival of bacteria (cfu/ml)	Log reduction	STDEV	Second treatment	Mean of survival of bacteria (cfu/ml)	Total log reduction	STDEV
Control	1.0×10^8	0	0.06	-			
10 sec laser	5.8×10^7	0.25	0.10	10 sec UV	7.7×10^6	1.14	0.24
10 sec laser	4.7×10^7	0.34	0.21	15 sec UV	5.7×10^6	1.27	0.30
11 sec laser	1.2×10^6	1.95	0.10	10 sec UV	7.2×10^4	3.16	0.35
11 sec laser	1.0×10^6	2.02	0.04	15 sec UV	2.8×10^4	3.58	0.53
10 sec UV	4.8×10^7	0.34	0.02	10 sec laser	1.6×10^7	0.82	0.10
15 sec UV	4.9×10^7	0.33	0.27	10 sec laser	2.6×10^6	1.60	0.66
10 sec UV	6.1×10^7	0.23	0.09	11 sec laser	5.2×10^5	2.30	0.40
15 sec UV	3.6×10^7	0.46	0.15	11 sec laser	1.7×10^4	3.78	0.20

Table 3-20. Killing effect of separate treatments on *M. luteus*

Treatment	Mean of log reduction (cfu/ml)	Treatments	Sum of mean of log reduction (cfu/ml)
10 sec UV alone	0.29	10 sec laser and 10 sec UV separately	0.59
15 sec UV alone	0.40	10 sec laser and 15 sec UV separately	0.70
10 sec laser alone	0.30	11 sec laser and 10 sec UV separately	2.27
11 sec laser alone	1.98	11 sec laser and 15 sec UV separately	2.38

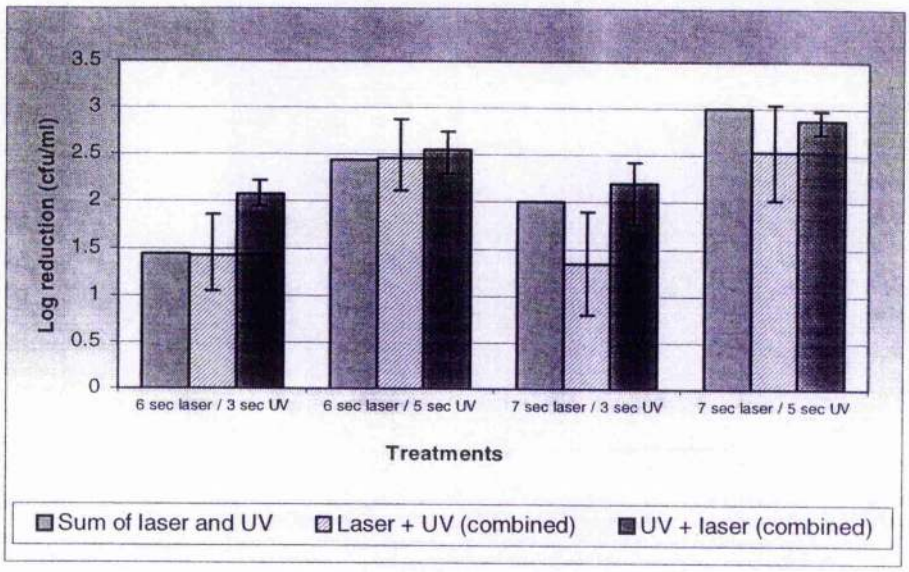


Figure 3-5. Comparison of killing effect of different sequential treatments on *S. putrefaciens* in saline suspension

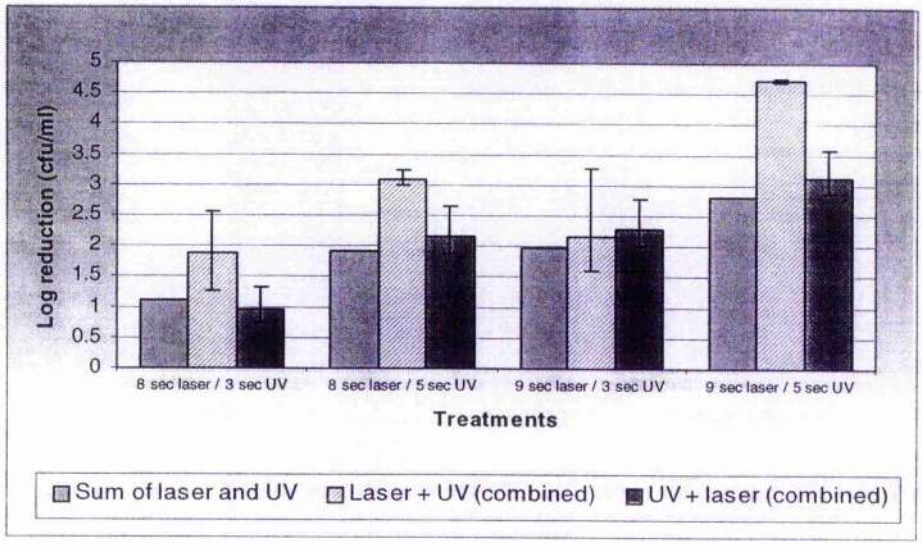


Figure 3-6. Comparison of killing effect of different sequential treatments on *P. fragi* in saline suspension

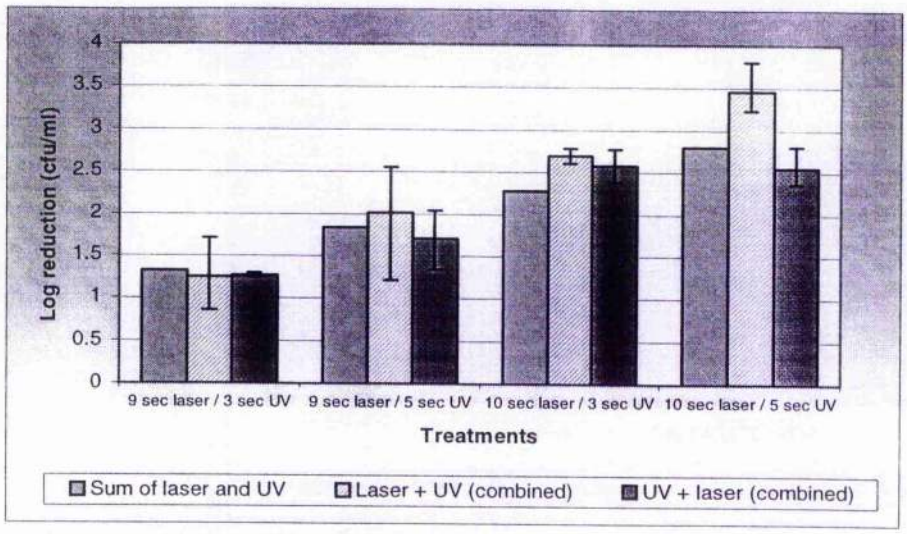


Figure 3-7. Comparison of killing effect of different sequential treatments on *E. coli (lux)* in saline suspension

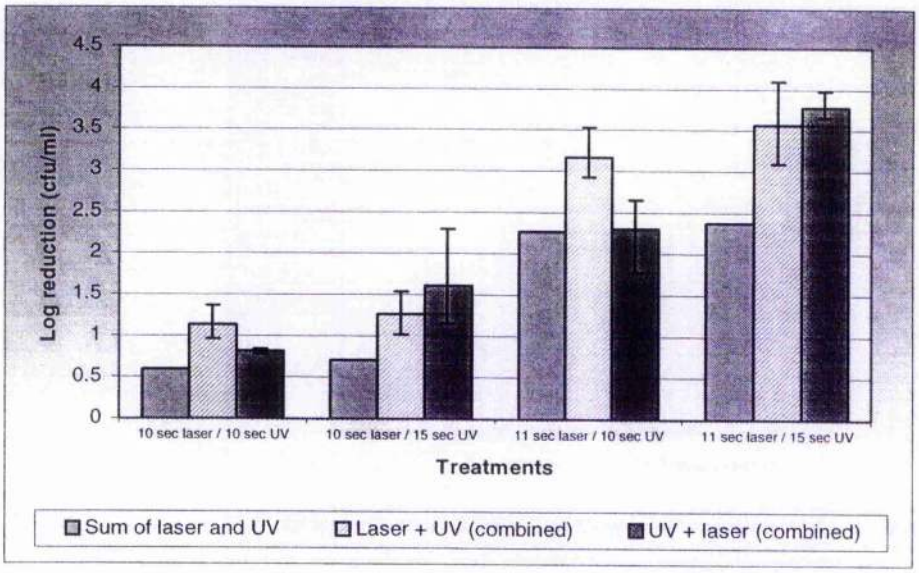


Figure 3-8. Comparison of killing effect of different sequential treatments on *M. luteus* in saline suspension

3.3 Sequential treatment of bacterial suspensions with combination of microwave, UV and laser radiation

In the last experiment a slight synergistic effect was observed when UV and laser treatments were combined sequentially and this effect was higher when higher treatment parameters were used. Next, the synergistic effect of the combination of UV and laser treatment after pre-treatment by microwave energy was investigated. The lowest energy density for the laser treatment and the two lowest UV exposure times for each bacterium were chosen to apply to the bacterial suspensions, after exposure to two low levels (10 and 15 sec exposure) of microwave energy. Also, the different orders of UV and laser treatment were investigated. The parameters used for each bacterium are shown below:

Bacteria	*S. putrefaciens*	*P. fragi*	*E. coli (lux)*	*M. luteus*
Laser parameters	6 sec (414.6 J/ cm^2)	8 sec (552.8 J/ cm^2)	9 sec (621.9 J/ cm^2)	10 sec (691 J/ cm^2)
UV parameters	3 sec/ 60 cm (1620 µWatt s/cm^2) 5 sec/ 60 cm (2700 µWatt s/cm^2)	3 sec/ 70 cm (1470 µWatt s/cm^2) 5 sec/ 70 cm (2450 µWatt s/cm^2)	3 sec/ 70 cm (1470 µWatt s/cm^2) 5 sec/ 70 cm (2450 µWatt s/cm^2)	10 sec/ 60 cm (5400 µWatt s/cm^2) 15 sec/ 60 cm (8100 µWatt s/cm^2)

Thus, suspensions of each bacterium were first exposed to microwave energy, then to either laser or UV, as the second treatment, then to either UV or laser to complete the combination of the three treatments. The killing by each treatment alone was also determined and the sum of the log reduction (cfu/ml) for each treatment alone was compared with the actual log reduction by the combination of the three treatments.

As shown in **Tables 3-21, 3-22, 3-23 and 3-24,** a large log reduction in viable counts was caused by the combined treatments in comparison to the sum of the individual treatments. The differences between these values for each bacterium are shown in the last column of each table (grey colour). Each table shows the control and the viable counts after the first, second and third treatment.

S. putrefaciens: As shown in the last experiment, despite the order, almost no synergistic effect was found when combined treatments of UV and laser were used. In this experiment, with 10s microwave pre-treatment, the final log reduction in the viable count was 2-3 logs, irrespective of whether UV or laser was given as the second treatment followed by laser or UV, respectively. However when the sum of the log reductions by the 3 treatments alone was calculated, the average reduction was 1-2 logs, therefore the sequential treatments gave about a 1 log greater reduction than the expected value.

With 15s of microwave treatment, which alone caused a significant reduction (2 log) in viability, the effect of the sequential treatment were even more dramatic. The sequential treatments all reduced the viability of the suspensions below the limit of detection (>6 log reduction) whereas the sum of the 3 treatments alone was 3.5- 4.5 log reduction. Thus the sequential treatment gave >1.5 to >2.5 greater log reduction than the sum of the individual treatments.

P. fragi: Differences between the log reductions in viability by the combined treatments in comparison to sum of the log reduction by the 3 treatments alone were even more than observed for the previous bacterium. 10s of microwave treatment gave <0.2 log decrease in viability but the final reduction in the viable counts was about 3 logs, in comparison to the sum of the log reduction by the 3 treatments alone, which was about 1.5 logs. Thus the combined treatment gave an increase of 1.5 about log reduction over the individual treatments.

Although, 15s of microwave treatment reduced the viability by about 1.5 logs, the final reduction in viability was similar to that seen with *S. putrefaciens* and below the limit of detection (>5.8 log reduction). Meanwhile the sum of the log reductions by the 3 treatments alone was between 2.5- 3.5 logs. So, the sequential treatment gave >2.3 to >3.2 greater log reduction in viability than the individual treatments.

E. coli (lux): With 10s of microwave energy, >0.2 log reduction in the viable count was measured and the differences between the final log reductions by the combined treatments in comparison to the sums of individual treatments were between 0.5 to 2 logs. When the bacterial suspensions were treated by microwave then laser then UV a greater

reduction in the viability was observed than if the treatments were microwave UV then laser. The mean of total log reduction by the 3 treatments combined achieved up to 5 log reduction, when the microwave treated suspensions were exposed to 9s laser followed by 5s UV. This was about 2 logs more than the reduction in viability by the sum of the three treatments alone. The same treatment energies but with a different sequence (microwave, UV then laser) gave about 3.5 log reduction in viability.

With 15s of microwave treatment, which alone caused 1 log reduction in viability, the sequential treatments with the order microwave, laser then UV, reduced the viability of the suspensions below the limit of detection (>5.8 log reduction) whereas the sum of the 3 treatments alone were about 2.6 (with 3 sec UV)-3.7 (with 5 sec UV) log reduction. Thus, more than 3 log greater reduction in the viability was induced by the combined treatments when compared with the sum of the three treatments alone. The sequential treatment order, microwave, UV then laser, gave a total of 5-5.5 log reduction in viability, which was about 2 logs more than the individual treatments alone.

M. luteus: With 10 s of microwave energy, <0.3 log reduction in the viability was observed. All sequences gave <2.74 log reduction in the viable counts, whereas the sums of the 3 treatments alone was 1.28 (with 10 sec UV) and 1.68 (with 15 sec UV) log reduction in the viability. So, except for the sequence, microwave then UV then laser, an increase of about 0.5 log reduction in the viable counts was apparent with the combination of 3 treatments in comparison to the sum of the log reductions by the individual treatments alone. After 10 s of microwave energy, 15 sec exposure to UV followed by 10 s of laser radiation, 2.74 log reduction in viability was observed which was about 1 log greater than the sum of the separate treatments. Although 15 s of microwave energy gave more than 0.5 log reduction in the viability, the final differences between the log reduction by combined treatments of microwave, laser then UV, and the sum of the log reduction by the 3 treatments alone was not greater than 0.5 log reduction in the viability. No difference in killing effect were calculated for microwave, UV then laser, in comparison to the sum of the separate treatments.

Comparisons of the killing effect of the different sequential treatments on selected bacteria are shown in **Figures 3-9, 3-10, 3-11 and 3-12.**

Table 3-21. Killing effect of combination of microwave, laser and UV on *S. putrefaciens* in saline suspension

Control

Mean of bacterial concentration (cfu/ml)	STDEV
5.8×10^8	0.22

First treatment

Treatment	Mean of survival of bacteria (cfu/ml)	Mean of log reduction (cfu/ml)	STDEV
10 sec microwave	3.8×10^8	0.18	0.11
15 sec microwave	5.1×10^8	2.06	0.01

Second treatment

	Treatment	Mean of survival of bacteria (cfu/ml)	Mean of log reduction (cfu/ml)	STDEV	Mean of cumulative log reduction (cfu/ml)
Pre-treated by 10 sec microwave	6 sec laser	2.2×10^7	1.24	0.43	1.43
	6 sec laser	2.4×10^7	1.20	0.48	1.39
	3 sec UV	4.5×10^6	1.93	0.36	2.11
	5 sec UV	4.2×10^5	2.96	0.28	3.15
Pre-treated by 15 sec microwave	6 sec laser	4.9×10^5	1.01	0.08	3.07
	6 sec laser	5.2×10^5	0.99	0.26	3.05
	3 sec UV	5.2×10^5	0.99	0.16	3.05
	5 sec UV	5.0×10^5	1.00	0.47	3.06

Third treatment

Treatment	Mean of survival of bacteria (cfu/ml)	Mean of log reduction (cfu/ml)	STDEV	Mean of total log reduction (cfu/ml)	Sum of three treatments alone	Differences
3 sec UV	6.3×10^5	1.54	0.39	2.96	1.62	1.27
5 sec UV	1.9×10^5	2.11	0.16	3.50	2.62	0.88
6 sec laser	8.2×10^5	0.74	0.29	2.85	1.62	1.23
6 sec laser	8.8×10^4	0.68	0.40	3.82	2.62	1.20
3 sec UV	<500	2.99	-	>6.07	3.50	>2.57
5 sec UV	<500	3.02	-	>6.07	4.50	>1.57
6 sec laser	<500	3.01	-	>6.07	3.50	>2.57
6 sec laser	<500	3.00	-	>6.07	4.50	>1.57

Table 3-22. Killing effect of combination of microwave, laser and UV on *P. fragi* in saline suspension

Control

Mean of bacterial concentration (cfu/ml)	STDEV
3.2×10^8	0.55

First treatment

Treatment	Mean of survival of bacteria (cfu/ml)	Mean of log reduction (cfu/ml)	STDEV
10 sec microwave	2.2×10^8	0.16	0.05
15 sec microwave	7.8×10^6	1.61	0.05

Second treatment

	Treatment	Mean of survival of bacteria (cfu/ml)	Mean of log reduction (cfu/ml)	STDEV	Mean of cumulative log reduction (cfu/ml)
Pre-treated by 10 sec microwave	8 sec laser	1.4×10^7	1.19	0.35	1.34
	8 sec laser	4.5×10^7	0.69	0.22	0.85
	3 sec UV	1.2×10^6	1.26	0.62	1.42
	5 sec UV	2.6×10^5	2.92	0.02	3.08
Pre-treated by 15 sec microwave	8 sec laser	3.0×10^3	3.41	0.36	5.02
	8 sec laser	4.0×10^3	3.29	0.24	4.90
	3 sec UV	2.5×10^4	2.49	0.19	4.10
	5 sec UV	5.0×10^4	2.19	0	3.80

Third treatment

Treatment	Mean of survival of bacteria (cfu/ml)	Mean of log reduction (cfu/ml)	STDEV	Mean of total log reduction (cfu/ml)	Sum of three treatments alone	Differences
3 sec UV	2×10^5	1.85	0.11	3.20	1.27	1.93
5 sec UV	1.8×10^5	2.39	0.07	3.24	2.08	1.16
6 sec laser	6.5×10^5	1.27	0.26	2.69	1.27	1.42
6 sec laser	6.2×10^4	0.63	0.31	3.71	2.08	1.63
3 sec UV	1.2×10^3	0.39	0.09	>5.41	2.61	2.80
5 sec UV	<500	>0.90	-	>5.80	3.53	>2.27
6 sec laser	<500	>1.70	-	>5.80	2.61	>3.19
6 sec laser	<500	>2.00	-	>5.80	3.53	>2.27

Table 3-23. Killing effect of combination of microwave, laser and UV on *E. coli* in saline suspension *(lux)*

Control

Mean of bacterial concentration (cfu/ml)	STDEV
3.7×10^8	0.04

First treatment

Treatment	Mean of survival of bacteria (cfu/ml)	Mean of log reduction (cfu/ml)	STDEV
10 sec microwave	2.6×10^8	0.15	0.13
15 sec microwave	3.3×10^7	1.05	0.56

Second treatment

	Treatment	Mean of survival of bacteria (cfu/ml)	Mean of log reduction (cfu/ml)	STDEV	Mean of cumulative log reduction (cfu/ml)
Pre-treated by 10 sec microwave	9 sec laser	3.2×10^7	0.91	0.07	1.06
	9 sec laser	4.5×10^7	0.76	0.24	0.92
	3 sec UV	1.3×10^7	1.30	0.23	1.45
	5 sec UV	8.2×10^5	2.50	0.30	2.65
Pre-treated by 15 sec microwave	9 sec laser	2.4×10^6	1.14	0.95	2.18
	9 sec laser	1.4×10^6	1.39	0.82	2.43
	3 sec UV	4.4×10^5	1.87	0.48	2.92
	5 sec UV	4.3×10^4	2.88	0.51	3.90

Third treatment

Treatment	Mean of survival of bacteria (cfu/ml)	Mean of log reduction (cfu/ml)	STDEV	Mean of total log reduction (cfu/ml)	Sum of three treatments alone	Differences
3 sec UV	2.2×10^5	2.16	0.36	3.22	2.13	1.09
5 sec UV	2.8×10^3	4.20	0.68	5.11	3.18	1.93
9 sec laser	2.2×10^5	1.77	0.28	3.22	2.13	1.09
9 sec laser	7.6×10^4	1.03	0.52	3.69	3.18	0.51
3 sec UV	<500	3.69	-	>5.87	2.66	>3.21
5 sec UV	<500	3.43	-	>5.87	3.71	>2.16
9 sec laser	1.7×10^3	2.41	0.33	5.33	2.66	2.67
9 sec laser	8.4×10^2	1.71	0.08	5.64	3.71	1.93

Table 3-24. Killing effect of combination of microwave, laser and UV on *M. luteus* in saline suspension

Control

Mean of bacterial concentration (cfu/ml)	STDEV
1.7×10^8	0.16

First treatment

Treatment	Mean of survival of bacteria (cfu/ml)	Mean of log reduction (cfu/ml)	STDEV
10 sec microwave	9.2×10^7	0.28	0.07
15 sec microwave	3.7×10^7	0.67	0.15

Second treatment

	Treatment	Mean of survival of bacteria (cfu/ml)	Mean of log reduction (cfu/ml)	STDEV	Mean of cumulative log reduction (cfu/ml)
Pre-treated by 10 sec microwave	10 sec laser	9.3×10^6	0.10	0.47	1.27
	10 sec laser	8.8×10^6	1.02	0.53	1.29
	10 sec UV	3.2×10^7	0.46	0.10	0.74
	15 sec UV	1.7×10^7	0.74	0.17	1.02
Pre-treated by 15 sec microwave	10 sec laser	1.1×10^7	0.54	1.12	1.22
	10 sec laser	1.2×10^7	0.48	1.09	1.15
	10 sec UV	2.4×10^7	0.18	0.40	0.86
	15 sec UV	1.1×10^7	0.52	0.71	1.19

Third treatment

Treatment	Mean of survival of bacteria (cfu/ml)	Mean of log reduction (cfu/ml)	STDEV	Mean of total log reduction (cfu/ml)	Sum of three treatments alone	Differences
10 sec UV	2.6×10^6	0.56	0.41	1.83	1.28	0.55
15 sec UV	1.3×10^6	0.83	0.64	2.13	1.68	0.45
10 sec laser	3.0×10^6	1.02	0.26	1.76	1.28	0.48
10 sec laser	3.2×10^5	1.72	0.28	2.74	1.68	1.06
10 sec UV	1.0×10^6	1.01	0.85	2.23	1.67	0.56
15 sec UV	4.7×10^5	1.42	1.69	2.57	2.07	0.50
10 sec laser	3.4×10^6	0.85	0.60	1.71	1.67	0.04
10 sec laser	1.7×10^6	0.82	0.80	2.08	2.07	0.01

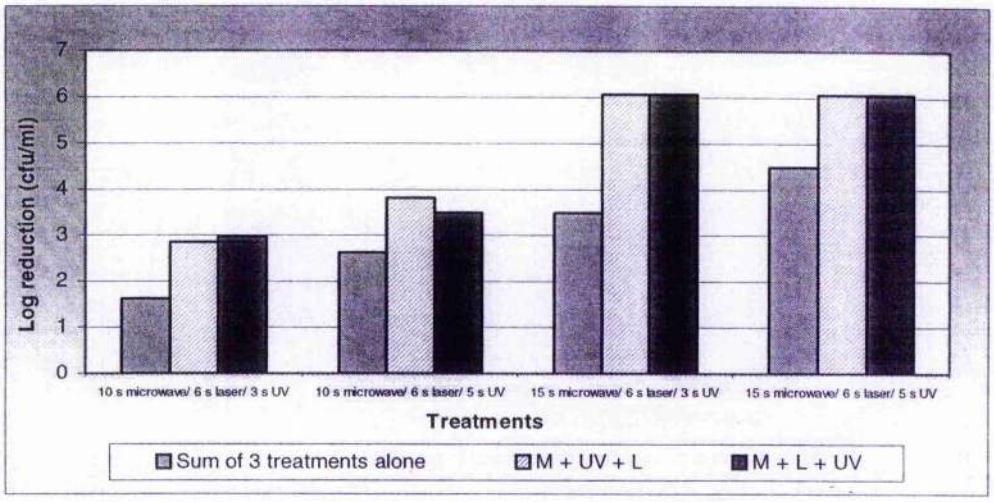


Figure 3-9. Comparison of killing effect of different sequential treatments on *S. putrefaciens* in saline suspension

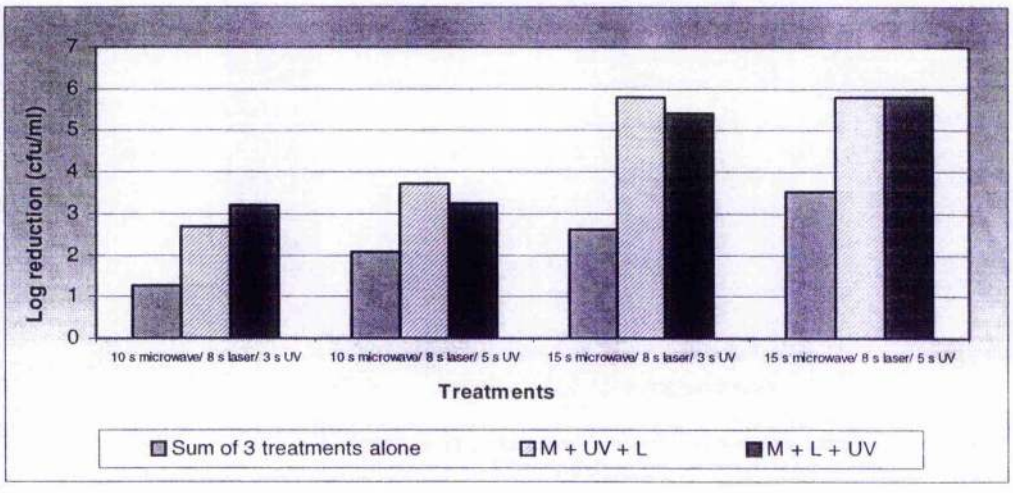


Figure 3-10. Comparison of killing effect of different sequential treatments on *P. fragi* in saline suspension

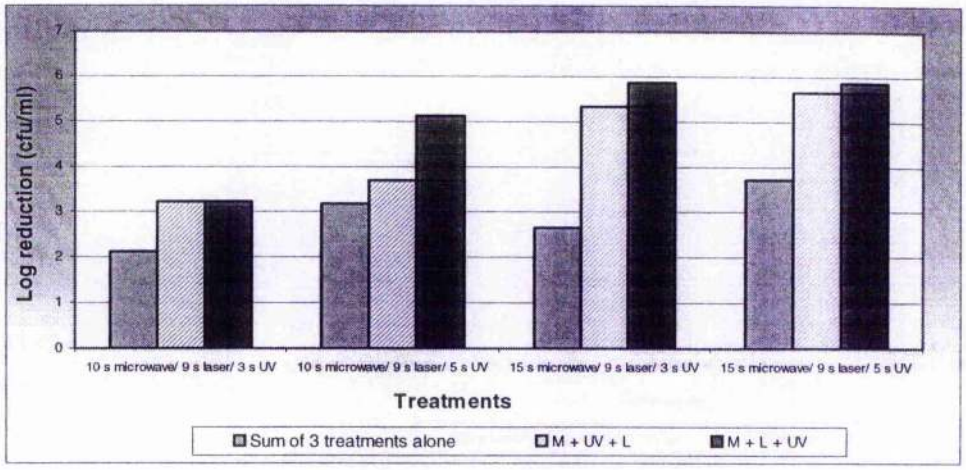


Figure 3-11. Comparison of killing effect of different sequential treatments on *E. coli (lux)* in saline suspension

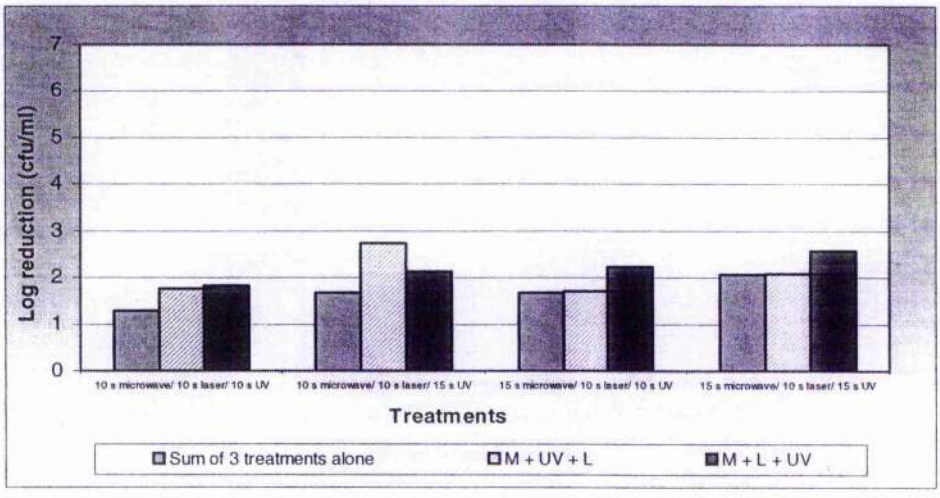


Figure 3-12. Comparison of killing effect of different sequential treatments on *M. luteus* in saline suspension

3.4 Further investigation of the killing effect of combination of laser, UV and microwave radiation with different treatment orders on *E. coli (lux)* and *P. fragi* in saline suspension

Further experiments were designed to determine the effect of the order of treatment for killing by the combination of laser, UV and microwave. *P. fragi* and *E. coli (lux)* were chosen for this experiment. These organisms were chosen as they demonstrated a favourable response to the combination treatments. Also, in previous experiments, for some orders of treatments, no colonies were counted after the last treatment and it was impossible to calculate the exact reduction in viability caused by the 3 treatments, so it was decided to use a higher initial concentration of bacteria. To simplify the experiments for each treatment, only 1 parameter was chosen to be used in combination with the other treatments. The chosen parameters are shown below:

Parameters used for *P. fragi*:			Parameters used for *E. coli (lux)*:		
UV	3 sec at 70 cm	1470 μW s/cm^2	UV	3 sec at 70 cm	1470 μW s/cm^2
Laser	8 sec	552.8 J/cm^2	Laser	9 sec	621.9 J/cm^2
Microwave	15 sec	800 W	Microwave	15 sec	800 W

All possible sequential treatments by the combination of UV, laser and microwave are shown below:

UV	▸ **Dilution**	▸ **Microwave**	▸ **Laser**
UV	▸ **Laser**	▸ **Dilution**	▸ **Microwave**
Laser	▸ **Dilution**	▸ **Microwave**	▸ **UV**
Laser	▸ **UV**	▸ **Dilution**	▸ **Microwave**
Dilution	▸ **Microwave**	▸ **UV**	▸ **Laser**
Dilution	▸ **Microwave**	▸ **Laser**	▸ **UV**

The above experiments were repeated four times for *P. fragi* (**Table 3-25**) and three times for *E. coli (lux)* (**Table 3-27**) to determine the statistical reliability of the results. The tables show the numbers of survivor and the calculation of the log reduction and

cumulative log reduction after each treatment. The mean of the log reduction, mean of the cumulative log reduction, mean of total log reduction and standard deviation of the 3 and 4 treatments for *P. fragi* and *E. coli (lux)* are shown in **Tables 3-26** and **3-28**, respectively. Also a summary of the results containing the mean of the total log reduction for each sequence, and the differences between the log reduction by the 3 treatments alone and combined treatments for both strains are shown in **Tables 3-29 and 3-30**.

The killing effect of the microwave radiation alone varied between 2-3.27 log reduction in viable count for *P. fragi* and between 0.17-0.81 for *E. coli (lux)*. This difference clearly shows the variable effect of this treatment and its effect on the results. The killing effect of the UV radiation alone varied between 0.03-0.39 log reductions in viability for *P. fragi* and between 0.02-0.27 for *E. coli (lux)*. These values, compared to previous results for the bacteria (**Tables 3-16 and 3-18**), were slightly reduced (by about 0.3-0.4 logs). Higher bacterial concentrations used in the current experiments may have caused these reductions. In contrast, the killing effect of the laser compared to previous results was increased. The killing effect of the laser on *P. fragi* was between 1.7- 2.53 log reduction in viable counts and between 1.77-2.7 for *E. coli (lux)*. Despite these matters, a synergistic effect on killing of the bacteria by combination of the three treatments was apparent for both strains. The differences between the log reduction in viable counts by the combination of treatments and the sum of the log reduction of the individual treatments alone was less than 1 log for *P. fragi* (**Table 3-29**), but for *E. coli* was between 0.42 to more than 3.16 logs (**Table 3-30**). It seems that the order of the treatment may be important in maximising the killing effect. The best sequence of the treatments for killing *P. fragi* was microwave, UV then laser, whereas the best order for killing *E. coli* (lux) was laser, microwave then UV.

Table 3-25. Results of killing effect of combination of microwave, laser and UV, in different orders, on *P. fragi* in saline suspension

Experiment 1:

Control: 1.5 x 10^{10} cfu/ml

	First treatment	Survivor bacteria (cfu/ml)	LR* cfu/ml	First or Second treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	CLD** cfu/ml	Second treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	CLD cfu/ml	Third treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	Total log reduction Cfu/ml
1	UV	(1 x 10^{10})	0.17	Dilution	(1.2 x 10^{8})	-	-	Microwave	(3 x 10^{4})	3.60	3.77	Laser	(1.2 x 10^{2})	2.40	6.17
2	UV	(8 x 10^{9})	0.27	Laser	(5 x 10^{6})	3.21	3.48	Dilution	(7 x 10^{4})	-	-	Microwave	(2.5 x 10^{3})	1.45	4.93
3	Laser	(3 x 10^{8})	1.70	Dilution	(2 x 10^{6})	-	-	Microwave	(3.5 x 10^{5})	0.76	1.93	UV	(9 x 10^{3})	1.59	3.52
4	Laser	(5 x 10^{7})	2.48	UV	(5 x 10^{6})	1.00	3.48	Dilution	(7.5 x 10^{4})	-	-	Microwave	(1 x 10^{2})	2.87	6.35
5	Dilution	(5 x 10^{8})	-	Microwave	(8 x 10^{5})	2.79	2.79	UV	(7.5 x 10^{4})	1.03	3.82	Laser	(6 x 10^{3})	1.10	4.92
6	Dilution	(4.2x10^{8})	-	Microwave	(1 x 10^{6})	2.62	2.69	Laser	(5 x 10^{4})	1.31	3.93	UV	(2 x 10^{3})	1.39	5.32

Experiment 2:

Control: 1 x 10^{10} cfu/ml

	First treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	First or Second treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	CLD cfu/ml	Second treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	CLD cfu/ml	Third treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	Total log reduction Cfu/ml
1	UV	(8.5 x 10^{9})	0.08	Dilution	(1 x 10^{8})	-	-	Microwave	(2.5 x 10^{5})	2.61	2.69	Laser	(6 x 10^{2})	2.62	5.31
2	UV	(8 x 10^{9})	0.10	Laser	(3.2 x 10^{8})	1.40	1.50	Dilution	(2.5 x 10^{6})	-	-	Microwave	(2 x 10^{4})	2.09	3.59
3	Laser	(6 x 10^{7})	2.23	Dilution	(5 x 10^{5})	-	-	Microwave	(7 x 10^{3})	1.85	4.08	UV	(1 x 10^{3})	0.84	4.92
4	Laser	(3 x 10^{7})	2.53	UV	(2.2 x 10^{7})	0.13	2.66	Dilution	(1.7 x 10^{5})	-	-	Microwave	(5 x 10^{2})	2.54	5.20
5	Dilution	(7.5 x 10^{8})	-	Microwave	(4 x 10^{5})	3.27	3.27	UV	(4 x 10^{4})	1.00	4.27	Laser	(2 x 10^{2})	2.30	6.57
6	Dilution	(5 x 10^{8})	-	Microwave	(2.5 x 10^{6})	2.30	2.30	Laser	(2.5 x 10^{5})	1.00	3.30	UV	(3 x 10^{3})	1.92	5.22

Continued

Experiment 3:

Control: 7.5×10^9 cfu/ml

	First treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	First or Second treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	CLD cfu/ml	Second treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	CLD cfu/ml	Third treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	Total log reduction Cfu/ml
1	UV	(7×10^9)	0.03	Dilution	(1.5×10^8)	-	-	Microwave	(5×10^4)	3.48	3.51	Laser	(1×10^3)	1.69	5.20
2	UV	(4×10^9)	0.27	Laser	(7.5×10^8)	0,73	1.00	Dilution	(2.5×10^6)	-	-	Microwave	(5×10^3)	2.70	3.70
3	Laser	(3×10^8)	1.40	Dilution	(2×10^6)	-	-	Microwave	(3.5×10^5)	0.76	2.16	UV	(2×10^4)	1.24	3.40
4	Laser	(2.5×10^8)	1.48	UV	(1.9×10^8)	0.09	1.57	Dilution	(2.5×10^7)	-	-	Microwave	(1×10^4)	3.39	4.96
5	Dilution	(7×10^8)	-	Microwave	(7×10^6)	2.00	2.00	UV	(4×10^5)	1.24	3.24	Laser	(2.5×10^4)	1.21	4.45
6	Dilution	(5×10^8)	-	Microwave	(2.5×10^6)	2.30	2.30	Laser	(2.5×10^5)	0.89	3.19	UV	(2.5×10^3)	2.11	5.30

Experiment 4:

Control: 1×10^9 cfu/ml

	First treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	First or Second treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	CLD cfu/ml	Second treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	CLD cfu/ml	Third treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	Total log reduction cfu/ml
1	UV	(5×10^8)	0.30	Dilution	(7.5×10^6)	-	-	Microwave	(2.5×10^5)	1.47	1.77	Laser	(5×10^2)	2.69	4.47
2	UV	(4×10^8)	0.39	Laser	(3×10^8)	0.12	0.52	Dilution	(3×10^6)	-	-	Microwave	(1×10^5)	1.47	1.96
3	Laser	(5×10^8)	0.30	Dilution	(5×10^6)	2.00	0.30	Microwave	(2.5×10^4)	2.30	2.60	UV	(1×10^3)	1.39	3.99
4	Laser	(7.5×10^8)	0.12	UV	(7×10^7)	1.03	1.15	Dilution	(7.5×10^5)	-	-	Microwave	(2.5×10^4)	1.47	2.62
5	Dilution	(3×10^8)	-	Microwave	(2.5×10^6)	2.08	2.08	UV	(2×10^5)	1.09	3.89	Laser	(7×10^3)	1.45	4.62
6	Dilution	(3×10^8)	-	Microwave	(2.5×10^6)	2.08	2.08	Laser	(2.5×10^4)	2.00	4.08	UV	(1.5×10^3)	1.22	5.30

* LR = Log reduction

** CLD = Cumulative log reduction

Parameters: UV, 3 sec at 70 cm (1470 μW s/cm^2); Nd:YAG laser, 8 sec (552.8 J/cm^2); Microwave, 15 sec (800 W)

Table 3-26 Mean of killing effect of combination of microwave, laser and UV, in different treatment orders, on *P. fragi* in saline suspension

Mean of Stock (control) 8.7×10^9 cfu/ml

▼

First treatment

Treatment	Mean of log reduction (cfu/ml)	STDEV*
UV	0.20	0.12
UV	0.54	0.12
Laser	1.43	0.81
Laser	1.78	1.13
Microwave	2.61	0.61
Microwave	2.33	0.22

▼

Second treatment

Treatment	Mean of log reduction (cfu/ml)	STDEV	Mean of cumulative log reduction (cfu/ml)	STDEV
Microwave	2.10	0.98	2.31	0.90
Laser	1.37	1.34	1.91	1.30
Microwave	1.25	0.78	2.68	0.86
UV	0.85	0.52	2.63	1.05
UV	1.00	0.11	3.61	0.51
Laser	1.44	0.50	3.77	0.44

▼

Third treatment

Treatment	Mean of log reduction (cfu/ml)	STDEV	Mean of total log reduction (cfu/ml)	STDEV
Laser	2.60	0.45	4.91	0.70
Microwave	1.56	0.59	3.47	1.21
UV	1.80	0.31	4.48	0.62
Microwave	2.02	0.81	4.65	1.56
Laser	1.74	0.54	5.35	0.97
UV	1.51	0.42	5.28	0.04

* STDEV = Standard deviation

Table 3-27. Results of killing effect of combination of microwave, laser and UV, in different orders, on *E. coli (lux)* in saline suspension

Experiment 1:

Control: 5.2×10^9 cfu/ml

	First treatment	Survivor bacteria (cfu/ml)	LR* cfu/ml	First or Second treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	CLD** cfu/ml	Second treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	CLD cfu/ml	Third treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	Total log reduction cfu/ml
1	UV	(5×10^9)	0.02	Dilution	(2.5×10^7)	-	-	Microwave	(2.5×10^6)	1.00	1.02	Laser	(1.5×10^4)	2.22	3.24
2	UV	(2.5×10^9)	0.32	Laser	(3×10^7)	1.92	2.24	Dilution	(4.5×10^5)	-	-	Microwave	(1.5×10^5)	0.51	2.75
3	Laser	(6×10^7)	1.94	Dilution	(5.5×10^5)	-	-	Microwave	(1×10^4)	1.74	3.68	UV	(<50)	>2.31	>5.99
4	Laser	(7.5×10^7)	1.84	UV	(3×10^6)	1.40	3.24	Dilution	(3.5×10^4)	-	-	Microwave	(5×10^3)	0.85	4.09
5	Dilution	(4.5×10^8)	-	Microwave	(2×10^8)	0.35	0.35	UV	(7×10^7)	0.46	0.81	Laser	(7×10^5)	2.00	2.81
6	Dilution	(4.5×10^8)	-	Microwave	(7×10^7)	0.81	0.81	Laser	(4.5×10^5)	2.19	3.00	UV	(3.5×10^3)	2.11	5.11

Experiment 2:

Control: 7.5×10^9 cfu/ml

	First treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	First or Second treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	CLD cfu/ml	Second treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	CLD cfu/ml	Third treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	Total log reduction Cfu/ml
1	UV	(5.5×10^9)	0.13	Dilution	(5×10^7)	-	-	Microwave	(8×10^6)	2.05	2.18	Laser	(4×10^4)	2.30	4.48
2	UV	(5×10^9)	0.18	Laser	(4×10^6)	3.09	3.27	Dilution	(1.5×10^4)	-	-	Microwave	(2.5×10^3)	0.78	4.05
3	Laser	(1.5×10^7)	2.70	Dilution	(5×10^4)	-	-	Microwave	(2×10^4)	0.39	3.09	UV	(<50)	>2.61	>5.70
4	Laser	(3.5×10^7)	2.33	UV	(1.5×10^7)	0.37	2.70	Dilution	(2×10^5)	-	-	Microwave	(1.5×10^3)	2.13	4.83
5	Dilution	(5.5×10^8)	-	Microwave	(1.7×10^8)	0.51	0.51	UV	(1.1×10^7)	1.19	1.70	Laser	(2×10^3)	1.74	3.44
6	Dilution	(5.5×10^8)	-	Microwave	(1.1×10^8)	0.70	0.70	Laser	(8×10^6)	1.50	2.20	UV	(1.2×10^3)	1.83	4.03

Continued

Experiment 3:

Control: 5.5 x 10^9 cfu/ml

	First treatment	Survivor bacteria (cfu/ml)	LR* cfu/ml	First or Second treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	CLD** cfu/ml	Second treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	CLD cfu/ml	Third treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	Total log reduction Cfu/ml
1	UV	(3 x 10^9)	0.27	Dilution	(5 x 10^7)	-	-	Microwave	(4 x 10^7)	0.09	0.36	Laser	(4 x 10^5)	2.00	2.36
2	UV	(5 x 10^9)	0.05	Laser	(1 x 10^8)	1.69	1.74	Dilution	(2 x 10^6)	-	-	Microwave	(1.5 x 10^5)	1.13	2.87
3	Laser	(9.5 x 10^7)	1.77	Dilution	(5 x 10^5)	-	-	Microwave	(2.5 x 10^4)	1.30	3.07	UV	(<50)	>2.70	>5.77
4	Laser	(5 x 10^7)	2.05	UV	(2 x 10^7)	0.39	2.44	Dilution	(4 x 10^5)	-	-	Microwave	(1.2 x 10^4)	1.76	4.20
5	Dilution	(6 x 10^8)	-	Microwave	(4 x 10^8)	0.17	0.17	UV	(1.7 x 10^7)	1.37	1.54	Laser	(3 x 10^5)	1.76	3.30
6	Dilution	(6 x 10^8)	-	Microwave	(4 x 10^8)	0.17	0.17	Laser	(2.5 x 10^7)	1.21	1.38	UV	(1.9 x 10^5)	2.12	3.50

* LR = Log reduction

** CLD = Cumulative log reduction

Parameters: UV, 3 sec at 70 cm (1470 μW s/cm^2); Nd:YAG laser, 9 sec (621.9 J/cm^2); Microwave, 15 sec (800 W)

Table 3-28 Mean of killing effect of combination of microwave, laser and UV, in different orders, on *E. coli (lux)* in saline suspension

Mean of Stock (control) 6.3×10^9 cfu/ml

▼

First treatment

Treatment	Mean of log reduction (cfu/ml)	STDEV
UV	0.14	0.12
UV	0.18	0.13
Laser	2.13	0.49
Laser	2.07	0.24
Microwave	0.34	0.17
Microwave	0.56	0.34

▼

Second treatment

Treatment	Mean of log reduction (cfu/ml)	STDEV	Mean of cumulative log reduction (cfu/ml)	STDEV
Microwave	1.04	0.98	1.18	0.92
Laser	2.23	0.75	2.41	0.78
Microwave	1.14	0.68	3.28	0.34
UV	0.72	0.58	2.79	0.40
UV	1.00	0.48	1.35	0.47
Laser	1.63	0.50	2.19	0.81

▼

Third treatment

Treatment	Mean of log reduction (cfu/ml)	STDEV	Mean of total log reduction (cfu/ml)	STDEV
Laser	2.17	0.15	3.36	1.06
Microwave	0.80	0.31	3.22	0.71
UV	2.54	0.20	>5.82	0.15
Microwave	1.58	0.65	4.37	0.39
Laser	1.83	0.14	3.18	0.33
UV	2.02	0.16	4.21	0.82

STDEV = Standard deviation

Table 3-29 Summary of killing effect of combination of microwave, laser and UV on *P. fragi*

Sequence of treatments	Total log reduction cfu/ml	STDEV	Difference*
Mic. ▷ UV ▷ Laser	5.35	0.97	0.91
Mic. ▷ Laser ▷ UV	5.28	0.04	0.84
UV ▷ Mic. ▷ Laser	4.91	0.70	0.47
Laser ▷ UV ▷ Mic.	4.65	1.56	0.21
Laser ▷ Mic. ▷ UV	4.48	0.62	0.04
UV ▷ Laser ▷ Mic.	3.47	1.21	-0.97
Sum of three treatments alone	4.44	-	-

* Difference between log reduction in viabilities caused by combination treatment and sum of three treatments alone

Table 3-30 Summary of killing effect of combination of microwave, laser and UV on *E. coli (lux)*

Sequence of treatments	Total log reduction cfu/ml	STDEV	Difference
Laser ▷ Mic. ▷ UV	>5.82	0.15	>3.16
Laser ▷ UV ▷ Mic.	4.37	0.39	1.71
Mic. ▷ Laser ▷ UV	4.21	0.82	1.55
UV ▷ Mic. ▷ Laser	3.36	1.06	0.7
UV ▷ Laser ▷ Mic.	3.22	0.71	0.56
Mic. ▷ UV ▷ Laser	3.18	0.33	0.52
Sum of three treatments alone	2.66	-	-

3.5 Treatment of saline suspensions of *E. coli (lux)* by combination of laser, UV and conventional heating with different treatment orders

In this investigation conventional heating was substituted for microwave radiation to investigate any synergistic effect of the three treatments on the killing of bacteria. The heating was supplied by a water bath and provided greater control of heating and reproducibility than the microwave. Parameters similar to those in the previous experiment were used. Because of the need to remove 10µl volumes from the treated suspensions (for the colony counting) and also to allow transfer of the suspensions to a different container, a starting volume 1.1 ml of suspension was used. Also, for more control of the UV exposure, the distance between the sample and lamps was increased to 80 cm allowing an increase in the exposure times. A preliminary experiment was done to determine the killing effect of conventional heating. A suspension of the bacterium was exposed to different temperatures for various times in a water bath. The results of this experiment are shown in **Table 3-31.** Almost no killing was observed at 45°C. A 0.06 log reduction in viability was observed after treating the suspension at 50°C for 5 min. Killing increased to 0.36 log reduction in viability after treatment of the bacterial suspension at 55°C for 5 min. The value increased to 0.69, 2.17, 2.39 and 2.87, respectively, for treatment of the bacterial suspension at 60°C for 2, 3, 4 and 5 min. On the bases of these results the parameters shown below were selected for investigation of the effect of combination of laser, UV and conventional heating on *E. coli (lux).*

UV	**5 sec at 80 cm**	**2300 µW s/cm^2**
Laser	**8 sec**	**552.8 J/cm^2**
Heating	**5 min**	**50 °C**

The experiment was repeated three times and the results are shown in **Table 3-32,** where the results of each treatment alone can be seen from the first and second parts of each table. The table shows the number of survivors and the log reduction and cumulative log reduction after each treatment. Also, the mean of the log reduction in viable counts by each treatment, mean of the cumulative log reduction and mean of the total log reduction

by the sequential treatments are shown in **Table 3-33**. A summary of the killing effect of the combination of the three treatments and a comparison of the log reduction in viability by the combined treatments and the 3 treatments alone can be see in **Table 3-34**.

Heating did not make a major reduction in the viable counts. The log reductions in the viability by the heat treatments were between 0-0.22 logs. Almost similar results were apparent for the laser treatments. The killing effect of UV on the bacterium was greater and between 0.15-1.22 log reduction in the viability but the mean of the results was around 0.7 logs. The standard deviation of the mean of the log reduction in viability generally increased, from the first to the second and third treatments (**Table 3-33**). A summary of the results (**Table 3-34**) showed that a synergistic effect was apparent when the combination of three treatments was used in comparison to the individual treatments. The differences were between 0.38 – 1.06 log reductions in viable counts. Although the results statistically were not significant, the mean of the log reduction in viability by different sequence showed that the order of the treatments might be important. The best order to kill the bacterium was laser, heating then UV and the least effective order was heating, UV then laser. The best and worst orders were the same as those seen with the combination of microwave, UV and laser. These two orders were chosen to investigate further under standard conditions.

Table 3-31. Killing effect of conventional heating on *E. coli (lux)* in saline suspension

Time (minutes)	45°C		50°C		55°C		60°C	
	Survivor bacteria cfu/ml	Log reduction	Survivor bacteria cfu/ml	Log reduction	Survivor bacteria cfu/ml	Log reduction	Survivor bacteria cfu/ml	Log reduction
2	1.5×10^9	0	1.5×10^9	0	1.5×10^9	0	3×10^7	1.69
3	1.5×10^9	0	1.4×10^9	0.02	1.4×10^9	0.02	1×10^7	2.17
4	1.5×10^9	0	1.4×10^9	0.02	1.2×10^9	0.09	6×10^6	2.39
5	1.4×10^9	0.02	1.3×10^9	0.06	6.5×10^8	0.36	2×10^6	2.87
Control	1.5×10^9 cfu/ml							

Table 3-32. Results of killing effect of combination of conventional heating, laser and UV, in different treatment orders, on *E. coli (lux)* in saline suspension

Experiment 1:

Control: 1×10^9 cfu/ml

	First treatment	Survivor bacteria (cfu/ml)	LR* cfu/ml	Second treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	CLD cfu/ml	Third treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	Total log reduction Cfu/ml
1	Heat	(1×10^9)	0.00	UV	(3.5×10^8)	0.45	0.45	Laser	(2.5×10^8)	0.15	0.19
2	Heat	(1×10^9)	0.00	Laser	(8×10^8)	0.09	0.09	UV	(8×10^7)	1.00	1.09
3	UV	(7×10^8)	0.15	Heat	(5×10^8)	0.15	0.30	Laser	(1×10^8)	0.54	0.84
4	UV	(4.5×10^8)	0.35	Laser	(3×10^8)	0.17	0.52	Heat	(1.5×10^8)	0.30	0.82
5	Laser	(1×10^9)	0.00	Heat	(2.5×10^8)	0.60	0.60	UV	(4×10^7)	0.79	1.40
6	Laser	(1×10^9)	0.00	UV	(2.5×10^8)	0.60	0.60	Heat	(9×10^7)	0.44	1.04

Experiment 2:

Control: 5.5×10^8 cfu/ml

	First treatment	Survivor bacteria (cfu/ml)	LR* cfu/ml	Second treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	CLD cfu/ml	Third treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	Total log reduction Cfu/ml
1	Heat	(3×10^8)	0.22	UV	(3×10^7)	1.00	1.22	Laser	(5×10^6)	0.78	2.00
2	Heat	(3.5×10^8)	0.15	Laser	(2×10^8)	0.24	0.39	UV	(1×10^6)	2.30	2.69
3	UV	(3×10^7)	1.22	Heat	(9×10^6)	0.52	1.74	Laser	(2.2×10^6)	0.61	2.35
4	UV	(1×10^8)	0.70	Laser	(2.5×10^7)	0.60	1.30	Heat	(4×10^6)	0.79	2.09
5	Laser	(5×10^8)	0.00	Heat	(4×10^8)	0.10	0.09	UV	(1.5×10^6)	2.42	2.52
6	Laser	(5×10^8)	0.00	UV	(2.5×10^6)	2.30	2.30	Heat	(2×10^6)	0.09	2.39

Continued

Experiment 3:

Control: 8 x 10^8 cfu/ml

	First treatment	Survivor bacteria (cfu/ml)	LR* cfu/ml	Second treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	CLD cfu/ml	Third treatment	Survivor bacteria (cfu/ml)	LR cfu/ml	Total log reduction Cfu/ml
1	Heat	(7.5 x 10^8)	0.03	UV	(1x 10^8)	0.87	0.90	Laser	(4.5 x 10^7)	0.35	1.25
2	Heat	(8x 10^8)	0.00	Laser	(5.5 x 10^8)	0.16	0.16	UV	(4.5 x 10^7)	1.08	1.24
3	UV	(1.4 x 10^8)	0.75	Heat	(1.2 x 10^8)	0.06	0.82	Laser	(4.5 x 10^7)	0.42	1.24
4	UV	(1.7 x 10^8)	0.67	Laser	(7.5 x 10^7)	0.35	1.03	Heat	(4.2 x 10^7)	0.25	1.28
5	Laser	(7 x 10^8)	0.06	Heat	(3.5 x 10^7)	1.30	1.36	UV	(2.1 x 10^7)	0.22	1.58
6	Laser	(7.5 x 10^8)	0.28	UV	(3.9 x 10^7)	1.28	1.31	Heat	(3.5x 10^7)	0.04	1.35

* LR = Log reduction

** CLD = Cumulative log reduction

Parameters: UV, 5 sec at 80 cm (2300 µW/cm^2); Nd:YAG laser, 8 sec (552.8 J/cm^2); Heating, 5 min (50 °C)

Table 3-33. Mean of killing effect of combination of conventional heating, laser and UV, in different treatment orders, on *E. coli (lux)* in saline suspension

Mean of Stock (control)
8×10^9cfu/ml

▼

First treatment

Treatment	Mean of log reduction (cfu/ml)	STDEV
Heat	0.08	0.12
Heat	0.05	0.09
UV	0.71	0.54
UV	0.57	0.19
Laser	0.02	0.03
Laser	0.09	0.16

▼

Second treatment

Treatment	Mean of log reduction (cfu/ml)	STDEV	Mean of cumulative log reduction (cfu/ml)	STDEV
UV	0.77	0.29	0.86	0.39
Laser	0.17	0.07	0.21	0.16
Heat	0.24	0.24	0.95	0.73
Laser	0.38	0.21	0.95	0.39
Heat	0.66	0.60	0.68	0.63
UV	1.39	0.85	1.40	0.85

▼

Third treatment

Treatment	Mean of log reduction (cfu/ml)	STDEV	Mean of total log reduction (cfu/ml)	STDEV
Laser	0.42	0.32	1.15	0.91
UV	1.46	0.73	1.68	0.88
Laser	0.58	0.14	1.48	0.78
Heat	0.45	0.30	1.40	0.65
UV	1.15	1.14	1.83	0.60
Heat	0.19	0.21	1.60	0.71

STDEV = Standard deviation

Table 3-34. Summary of killing effect of combination of conventional heating, laser and UV on *E. coli (lux)*

Sequence of treatments	Total log reduction cfu/ml	STDEV	Difference*
Laser ▷ Heat ▷ UV	1.83	0.91	1.06
Heat ▷ Laser ▷ UV	1.68	0.88	0.91
Laser ▷ UV ▷ Heat	1.60	0.78	0.83
UV ▷ Heat ▷ Laser	1.48	0.65	0.71
UV ▷ Laser ▷ Heat	1.40	0.60	0.63
Heat ▷ UV ▷ Laser	1.15	0.71	0.38
Sum of three treatments alone	0.77	-	-

* Difference between log reduction in viabilities caused by combination treatment and sum of three treatments alone

3.5.1 Standardisation of the temperature of bacterial suspensions between treatments

One possible reason why the sequential treatment gave greater killing than expected from the sum of the individual treatments is that the heating effect by the laser, and microwave or conventional heating could be cumulative and the bacterial suspension reached a higher final temperature when the treatments were combined. This was thought to be unlikely because of the small volume involved and the time elapsed between treatments, allowing cooling to take place. However, experiments were designed where the sample temperatures were standardised at 25°C at the start of the experiment and cooled to 25°C in a water bath after each treatment and before applying the subsequent treatment. In the previous experiment, UV was the last treatment in the optimum killing order and laser was last in the least effective order. It was decided, therefore to compare the killing effect of UV and laser radiation on bacteria by using UV or laser alone, or after other treatments, under standard conditions. Below is the experimental design for the best and worst order, in standard conditions.

For the best order:

Suspension at 25°C > ---------- > UV > 25°C

Suspension at 25°C > ---------- ---- > Heat > 25°C > UV > 25°C

Suspension at 25°C > Laser > 25°C----------> UV > 25°C

Suspension at 25°C > Laser > 25°C > Heat > 25°C > UV > 25°C

For the worst order:

Suspension at 25°C > ---------- >Laser > 25°C

Suspension at 25°C > ---------- > UV > 25°C > Laser > 25°C

Suspension at 25°C > Heat > 25°C---------->Laser > 25°C

Suspension at 25°C > Heat > 25°C > UV > 25°C > Laser > 25°C

The experiment was done on *E. coli (lux)*. The same treatment parameters were used as in the previous experiment and are shown below for convenience. Each treatment was done three times.

UV	**5 sec at 80 cm**	**2300 μW s/cm^2**
Laser	**8 sec**	**552.8 J/cm^2**
Heating	**5 min**	**50 °C**

The results are shown in **Table 3-35** for the previously determined best order of killing and in **Table 3-36** for the worst order. It is noticeable that the killing effects of the UV and laser were greater when they were used after the other treatments. One interesting result was observed for UV killing. When it was used after the laser, the killing effect was almost double that seen after heating. The killing effect of the laser and heat were similar for both experiments (series experiment 2 and 3 in **Table 3-35**). These results suggest that a different killing mechanism exists for heat and laser.

Under the conditions of the experiment, the sum of the log reduction in viability by the individual treatments was 0.84 (**Table 3-35**), but the value was increased to 1.77 logs (0.93 logs higher) when the same parameters were used in combination. In the other treatment order (**Table 3-36**), the sum of the three treatments alone was 0.56 logs but 1.06 logs after the combined treatment, which was 0.5 logs higher. The difference between the best and the worst orders of treatment was 0.71 logs. However, in these experiments the killing effect of conventional heating in both treatment series was variable, ranging from 0.03-0.26 log reduction in viable. Thus, it was decided to treat the samples by heat at the same time to eliminate variable factors that may have caused this difference.

The experiment was repeated with a minor change, where both series of samples were placed into the water bath at same time. Also, after heating, all the samples were treated to UV at the same time, but in a random order. The results are shown in **Table 3-37.** The killing effect of conventional heating in both series was almost the same at 0.12 log reduction in the viable counts, and the difference between the best and worst orders was similar to the previous experiment, about 0.7 log.

It was concluded that order of treatment (laser > heat > UV) was consistently and significantly better (0.7 logs) than the order (heat > UV > laser) in reducing the viable counts of *E. coli (lux)* in saline suspension (P<0.01), and there appeared to be a synergistic effect on killing the bacterium with the sequential treatments, compared to the sum of the individual treatments alone.

A similar experiment was done on *L. monocytogenes* to find out the killing effect of each treatment alone or in combination on the bacterium. The order (laser > heat > UV) and (heat > UV > laser) treatment was also investigated, but colony counts were only made before and after each complete treatment. The parameters below were used for treatment of *L. monocytogenes:*

Laser	**9 sec**	**621.9 J/ cm^2**
UV	**10 sec / 80 cm**	**4600 µW s/cm^2**
Conventional heating	**5 min**	**55°C**

Table 3-38 shows the surviving bacteria after each experiment, the mean number of the surviving bacteria (cfu/ml), the mean of the log reduction by each sequential treatment and finally the differences between the log reductions in viability by the combined treatments in comparison to the sum of the log reduction by the individual treatments. It can be seen that the UV treatment gave 0.5 log reduction in viability, whereas the laser and heating treatments were similar to each other at about 0.2 logs. Although in contrast to *E. coli (lux),* the difference between the best treatment order and the worst was about 0.2 logs, which was less than the value for *E. coli* (0.7 log) in the last experiment. The killing effect for the order L+H+UV was significantly higher than the order H+UV+L (P<0.01). It should be noted that the treatment parameters were different for the bacteria, because the sensitivity of each bacterium was different to each parameter.

Table 3-35. Comparison of killing effect of UV alone and UV treatment after laser, heat and laser plus heat on *E. coli (lux)* in saline suspension

		Laser		Heat		Mean of cumulative log reduction cfu/ml	UV		Mean of total log reduction cfu/ml
		Survivor bacteria cfu/ml	Mean of log reduction cfu/ml	Survivor bacteria cfu/ml	Mean of log reduction cfu/ml		Survivor bacteria cfu/ml	Mean of log reduction cfu/ml	
Control: 9×10^8 cfu/ml	Exp. 1	-	-	-	-	-	4×10^8	0.36	0.36
	Exp. 2	-		-			3.7×10^8		
	Exp. 3	-		-			4.2×10^8		
	Mean	-		-			3.9×10^8		
	Exp. 1	-	-	6×10^8	0.26	0.26	1.7×10^8	0.50	0.76
	Exp. 2	-		4×10^8			2×10^8		
	Exp. 3	-		5×10^8			1×10^8		
	Mean	-		5×10^8			1.5×10^8		
	Exp. 1	5×10^8	0.26	-	-	-	6×10^7	0.91	1.17
	Exp. 2	5.2×10		-			7.5×10		
	Exp. 3	4.5×10^8		-			5×10^7		
	Mean	4.9×10^8		-			6.1×10^7		
	Exp. 1	6.5×10^8	0.18	4×10^8	0.35	0.53	1×10^7	1.24	1.77
	Exp. 2	6.2×10^8		2×10			1.5×10^7		
	Exp. 3	5.1×10^8		2×10^8			2×10^7		
	Mean	5.9×10^8		2.6×10^8			1.5×10^7		
	Sum of killing effect of three treatments alone							0.84 log (cfu/ml)	
	Difference between killing effect of combined treatment and sum of individual treatment							0.93 log (cfu/ml)	

Table 3-36. Comparison of killing effect of laser alone and laser treatment after UV, heat and heat plus UV on *E. coli (lux)* in saline suspension

		Heat		UV		Mean of cumulative log reduction cfu/ml	Laser		Mean of total log reduction cfu/ml
		Survivor bacteria cfu/ml	Mean of log reduction cfu/ml	Survivor bacteria cfu/ml	Mean of log reduction cfu/ml		Survivor bacteria cfu/ml	Mean of log reduction cfu/ml	
Control: 9×10^8 cfu/ml	Exp. 1	-	-	-	-	-	8×10^8	0.12	0.12
	Exp. 2	-		-			7.5×10^8		
	Exp. 3	-		-			5×10^8		
	Mean	-		-			6.8×10^8		
	Exp. 1	-	-	6×10^8	0.41	0.41	3×10^8	0.28	0.69
	Exp. 2	-		4×10^8			1.5×10^8		
	Exp. 3	-		5×10^8			1×10^8		
	Mean	-		5×10^8			1.8×10^8		
	Exp. 1	9×10^8	0.03	-	-	-	6×10^8	0.24	0.27
	Exp. 2	7.5 x 10		-			4.5×10^8		
	Exp. 3	9×10^8		-			4×10^8		
	Mean	8.5×10^8		-			4.8×10^8		
	Exp. 1	8×10^8	0.03	3.5×10^8	0.38	0.41	6×10^7	0.65	1.06
	Exp. 2	8×10^8		3 x 10			7.5×10^7		
	Exp. 3	9×10^8		4×10^8			1×10^7		
	Mean	8.3×10^8		3.5×10^8			7.8×10^7		
	Sum of killing effect of three treatments alone							0.56 log (cfu/ml)	
	Difference between killing effect of combined treatment and sum of individual treatment							0.50 log (cfu/ml)	

Table 3-37. Comparison of the best and worst treatment sequences of combinations of UV, laser and conventional heating on killing *E. coli (lux)* in saline suspension

	Survivor bacteria (cfu/ml)	Mean of log reduction (cfu/ml)
Control	6.7×10^8	0

The best order	Laser			Heating			UV		
		Survivor bacteria (cfu/ml)	Mean of log reduction (cfu/ml)	Survivor bacteria (cfu/ml)	Mean of log reduction (cfu/ml)	Cumulative log reduction (cfu/ml)	Survivor bacteria (cfu/ml)	Mean of log reduction (cfu/ml)	Total log reduction (cfu/ml)
	Exp. 1	5.7×10^8		4.5×10^8			8.5×10^5		
	Exp. 2	5×10^8		4.3×10^8			9.5×10^5		
	Exp. 3	6×10^8	0.08	3×10^8	0.15	0.23	8.3×10^5	2.65	2.89
	Mean	5.5×10^8		3.9×10^8			8.7×10^5		

The worst order	Heating			UV			Laser		
		Survivor bacteria (cfu/ml)	Mean of log reduction (cfu/ml)	Survivor bacteria (cfu/ml)	Mean of log reduction (cfu/ml)	Cumulative log reduction (cfu/ml)	Survivor bacteria (cfu/ml)	Mean of log reduction (cfu/ml)	Total log reduction (cfu/ml)
	Exp. 1	5.5×10^8		9×10^6			3×10^6		
	Exp. 2	4×10^8		2×10^7			6×10^6		
	Exp. 3	6×10^8	0.12	1.2×10^7	1.59	1.71	4×10^6	0.48	2.19
	Mean	5.1×10^8		1.3×10^7			4.3×10^6		
Control		6.7×10^8	0						

Summary		
Laser > Heat > UV	**Heat > UV > Laser**	**Differences in total log reduction (cfu/ml)**
Total log reduction	**Total log reduction**	
2.89	**2.19**	**0.70**

Table 3-38. Killing effect of sequential treatments on *L. monocytogenes* in saline suspension

No	Treatment order	Experiment 1 Survivior bacteria cfu/ml	Experiment 2 Survivior bacteria cfu/ml	Experiment 3 Survivior bacteria cfu/ml	Mean Survivior bacteria cfu/ml	Mean of log reduction cfu/ml	STDEV*	Differences from sums of individual treatments
1	Control				4.5×10^8			
2	UV	1.7×10^8	1.5×10^8	1.6×10^8	1.6×10^8	0.50	0.03	-
3	Laser	2.5×10^8	3.5×10^8	2.3×10^8	2.7×10^8	0.21	0.09	-
4	Heat	2.0×10^8	2.5×10^8	3.5×10^8	2.6×10^8	0.22	0.12	-
5	Laser + UV	7.2×10^7	8.9×10^7	8.0×10^7	8.0×10^7	0.75	0.04	0.75-0.71=0.04
6	UV + laser	1.2×10^8	9.0×10^7	9.2×10^8	1.0×10^8	0.65	0.07	0.65-0.71=-0.06
7	Heat + UV	5.1×10^7	5.5×10^7	3.0×10^7	4.5×10^7	1.01	0.14	1.01-0.72=0.29
8	Heat + laser	5.0×10^7	3.2×10^7	3.5×10^7	3.9×10^7	1.07	0.10	1.07-0.43=0.58
9	Laser + Heat + UV	1.6×10^7	1.5×10^7	1.2×10^7	1.4×10^7	1.50	0.06	1.50-0.93=0.57
10	Heat + UV + laser	2.0×10^7	2.2×10^7	2.5×10^7	2.2×10^7	1.30	0.05	1.30-0.93=0.37
		Difference between the best and worst order in killing the bacterium = 0.20 log cfu/ml						

* Standard deviation

3.5.2 Effect of more severe treatment conditions on the killing effect of sequentially combined treatments of UV, laser and conventional heating on *E. coli (lux)* in saline suspension

Again, as described in section 3.5.1, sample temperatures were standardised at 25°C at the start of the experiment and cooled to 25°C in a water bath after each treatment and before applying the subsequent treatment. In the previous experiment, about 0.7 logs difference in killing of *E. coli (lux)* was found between the best and worst order of treatments. More severe treatment conditions were next used to find out if the synergistic effect of the combination of treatments on *E. coli (lux)* could be increased. Also, the differences between the best and worst orders were re-examined. The treatment parameters are shown below:

Parameter set 1*:		Parameter set 2:		Parameter set 3:	
8 sec	Laser 552.8 J/cm²	9 sec	Laser 621.9 J/cm²	9 sec	Laser 621.9 J/cm²
5 min	Conventional heating (50°C)	5 min	Conventional heating (55°C)	5 min	Conventional heating (55°C)
5 sec	UV radiation 2300 µW s/cm²	8 sec	UV radiation 3680 µW s/cm²	10 sec	UV radiation 4600 µW s/cm²

* Already done in section 3.5.1

The results are shown in **Table 3-39**. Interestingly, the differences between the best and worst orders of treatments increased when the more severe conditions were used. For the first set of parameters (already done in section 3.5.1), a difference of 0.7 log reduction in viability between the two orders was apparent ($P<0.01$). This difference increased to 1.15 and was significant ($P<0.001$) and 1.3 ($P<0.001$) for the second and third set of parameters respectively.

3.5.3 Killing effect of the best and worst sequences of combination of UV, laser and conventional heating on selected bacteria

In this experiment, sample temperatures were standardised at 25°C at the start and between treatments. The above data showed the importance of the order of the three treatments on killing *E. coli (lux)* and *L. monocytogenes*. The effect on other bacteria was

investigated with the best and worst orders of combined treatments. Based on the sensitivity of each bacterium to the treatments, the parameters below were chosen for each strain:

Parameter set for *P. fragi*:		Parameter set for *S. putrefaciens*:		Parameter set for *M. luteus*:	
8 sec	laser 552.8 J/cm^2	7 sec	laser 483.7 J/cm^2	9 sec	laser 621.9 J/cm^2
5 min	conventional heating (50°C)	5 min	conventional heating (50°C)	5 min	conventional heating (55°C)
5 sec	UV at 80 cm 2300 μW s/cm^2	5 sec	UV at 80 cm 2300 μW s/cm^2	20 sec	UV at 80 cm 9200 μW s/cm^2

The results are shown in **Table 3-40.** Differences between the best and worst order for *S. putrefaciens*, *P. fragi* and *M. luteus* were significant and 0.72 ($P<0.05$), 0.33 ($P<0.001$) and 0.54 ($P<0.001$) log reduction, respectively. Although the difference between the parameters chosen for each bacterium should be borne in mind, it seemed that the best and worst order may be slightly different for each bacterium.

Table 3-39. Differences of killing effect of the best and worst sequences of combination of UV, laser and conventional heating with different parameters on *E. coli (lux)* in saline suspension

	Laser > Heat > UV (best order)						Heat > UV > Laser (worst order)						Difference between orders log reduction (cfu/ml)
		Experiment 1	Experiment 2	Experiment 3	Mean	STDEV*		Experiment 1	Experiment 2	Experiment 3	Mean	STDEV*	
Set parameter 1	Start cfu/ ml	6.7×10^8	6.7×10^8	6.7×10^8	6.7×10^8	0	Start cfu/ ml	6.7×10^8	6.7×10^8	6.7×10^8	6.7×10^8	0	0.70**
	Finish cfu/ ml	8.5×10^5	9.5×10^5	8.3×10^5	8.7×10^5	0.03	Finish cfu/ ml	3×10^6	6×10^6	4×10^6	4.3×10^6	0.16	
	Log reduction cfu/ ml	2.90	2.85	2.91	2.89		Log reduction cfu/ml	2.34	2.04	2.21	2.19		
Set parameter 2	Start Cfu/ ml	2×10^9	2×10^9	2×10^9	2×10^9	0	Start cfu/ ml	2×10^9	2×10^9	2×10^9	2×10^9	0	1.15
	Finish cfu/ ml	5.1×10^5	6.2×10^4	7.1×10^5	4.2×10^5	0.42	Finish cfu/ ml	6.1×10^6	5.9×10^6	6×10^6	6×10^6	0.01	
	Log reduction cfu/ml	3.60	4.25	3.46	3.68		Log reduction cfu/ml	2.52	2.53	2.52	2.53		
Set parameter 3	Start cfu/ ml	1×10^9	1×10^9	1×10^9	1×10^9	0	Start cfu/ ml	1×10^9	1×10^9	1×10^9	1×10^9	0	1.30
	Finish cfu/ ml	2.1×10^4	4×10^4	3×10^4	3×10^4	0.15	Finish cfu/ ml	6×10^5	6.5×10^5	5.5×10^5	6×10^5	0.04	
	Log reduction cfu/ml	4.69	4.39	4.52	4.52		Log reduction cfu/ml	3.22	3.18	3.26	3.22		

* Standard deviation

** Data from previous experiment

Table 3-40. Differences of killing effect of the best and worst sequences of combination of UV, laser and conventional heating on selected bacteria in saline suspension

Bacteria	Laser > Heat > UV						Heat > UV > Laser						Difference between orders, log reduction (cfu/ml)
		Experiment 1	Experiment 2	Experiment 3	Mean	Log STDEV*		Experiment 1	Experiment 2	Experiment 3	Mean	SD*	
E. coli	Start Cfu/ ml	6.7×10^8	6.7×10^8	6.7×10^8	6.7×10^8	0	Start Cfu/ ml	6.7×10^8	6.7×10^8	6.7×10^8	6.7×10^8	0	0.7**
	Finish Cfu/ml	8.5×10^5	9.5×10^5	8.3×10^5	8.7×10^5	0.032	Finish Cfu/ml	3×10^6	6×10^6	4×10^6	4.3×10^6	0.17	
	Log reduction	2.9	2.85	2.91	2.89		Log reduction	2.34	2.04	2.21	2.19		
S. putrefaciens	Start Cfu/ ml	1×10^9	1×10^9	1×10^9	1×10^9	0	Start Cfu/ ml	1×10^9	1×10^9	1×10^9	1×10^9	0	0.72
	Finish Cfu/ml	2.5×10^2	5×10^2	2.5×10^2	3.3×10^2	0.17	Finish Cfu/ml	2.5×10^3	1×10^3	1.5×10^3	1.7×10^3	0.20	
	Log reduction	6.60	6.30	6.60	6.48		Log reduction	5.60	6.00	5.82	5.76		
M. luteus	Start Cfu/ ml	1×10^9	1×10^9	1×10^9	1×10^9	0	Start Cfu/ ml	1×10^9	1×10^9	1×10^9	1×10^9	0	0.54
	Finish Cfu/ml	3×10^7	3.2×10^7	3.5×10^7	3.2×10^7	0.04	Finish Cfu/ml	1.2×10^8	1×10^8	1.2×10^8	1.1×10^8	0.05	
	Log reduction	1.52	1.50	1.45	1.50		Log reduction	0.92	1.00	0.92	0.96		
P. fragi	Start Cfu/ ml	2×10^9	2×10^9	2×10^9	2×10^9	0	Start Cfu/ ml	2×10^9	2×10^9	2×10^9	2×10^9	0	0.33
	Finish Cfu/ml	7×10^7	6×10^7	7×10^7	6.7×10^7	0.04	Finish Cfu/ml	1.5×10^8	1.4×10^8	1.2×10^8	1.4×10^8	0.05	
	Log reduction	1.45	1.52	1.45	1.49		Log reduction	1.12	1.15	1.22	1.16		
L. monocytogenes	Start Cfu/ ml	4.5×10^8	4.5×10^8	4.5×10^8	4.5×10^8	0	Start Cfu/ ml	4.5×10^8	4.5×10^8	4.5×10^8	4.5×10^8	0	0.20**
	Finish Cfu/ml	1.6×10^7	1.5×10^7	1.2×10^7	1.4×10^7	0.06	Finish Cfu/ml	2.0×10^7	2.2×10^7	2.5×10^7	2.2×10^7	0.05	
	Log reduction	1.45	1.48	1.57	1.50		Log reduction	1.35	1.31	1.25	1.30		

* Standard deviation

** Data from previous experiments

3.6 Treatment of bacteria on agar plates with ozone

A suspension for each strain was made (sections 2.1.3.1 and 2.1.3.2) and colony counts were made to determined the bacterial concentration. The results are show below:

Bacteria	Concentration cfu/ml
S. typhimurium	8×10^6
L. monocytogenes	6×10^6
S. aureus	4×10^6
E. coli (lux)	4×10^6
C. jejuni	2.4×10^6

100 µl of each suspension was pipetted onto the surface of agar plates and spread as described in section 2.1.3.4. The plates were then placed into the treatment chamber (Figure 2-2) and treated for 2, 5, 10 and 15 min (section 2.3.10). The results are shown in **Tables 3-41, 3-42, 3-43, 3-44 and 3-45** for each bacterium, respectively. In **Figures 3-13, 3-14, 3-15, 3-16** and **3-17**, the log reductions after different exposure times are shown. It can be seen, except for *E. coli* (in one case), that ozonation of the plates for 2 minutes did not give any reduction in the viable counts in other strains. With longer treatments, however the results consistently showed that ozone was effective in killing. With all of the bacteria, viability was reduced with treatment time. After 15 minutes of treatment, the log reductions in viable counts for the three Gram-negative bacteria were 3.7 for *S. typhimurium*, 3.6 for *E. coli* and 3.8 for *C. jejuni*. For the Gram-positive bacteria, the log reductions in viable counts after ozonation for 15 min were 2.9 and 3.3 for *L. monocytogenes* and *S. aureus*, respectively. **Figure 3-18** shows comparison of cfu counts of the bacteria after 10 and 15 min ozonation on agar plates.

The Gram-positive bacteria were only slightly more resistant to ozonation than the Gram-negative bacteria. Looking at the spatial distribution of the colonies growing on the plates of all bacteria treated for 2 min and 15 min, it was observed that the killing effect by ozone was not homogenous with short treatment times. In all experiments at 2 min,

plates Q2 and Q4, which were positioned closer to the inlet of the ozone gas, showed more extensive clearing than plates Q1 and Q3, which were located at the far end of the chamber. This apparent non-homogenous distribution of the ozone gas appears to be less significant for longer treatment periods where the gas concentration was believe to be more uniform. **Figure 3-19** shows the killing effect of ozonation after 2 min on *S. typhimurium*. The results clearly indicated that in the case of treating the plate with ozone for short durations, the in-flow of gas was uneven, which resulted in the plates with an uneven distribution of colonies. The killing effect of ozonation for 15 min on *S. typhimurium* is shown in **Figure 3-20.**

Table 3-41. Killing effect of ozone on *S. typhimurium* on agar plates

Treatment	2 min		5 min		10 min		15 min	
Location in chamber	*Q1*	*Q2*	*Q1*	*Q2*	*Q1*	*Q2*	*Q1*	*Q2*
Survivor bacteria cfu/plate	TM*	TM	TM	9.74×10^2	2.15×10^3	5.78×10^2	1.24×10^3	2.06×10^2
Log reduction cfu/plate	-	-	-	2.91	2.57	3.14	2.81	3.59
Location in chamber	*Q3*	*Q4*	*Q3*	*Q4*	*Q3*	*Q4*	*Q3*	*Q4*
Survivor bacteria cfu/plate	TM	TM	TM	1.35×10^3	3.27×10^3	4.33×10^2	1.86×10^3	1.61×10^2
Log reduction cfu/plate	-	-	-	2.77	2.39	3.26	2.63	3.7
Control: 8.00×10^5 (cfu/plate)								

*TM: Too many to be counted

Table 3-42. Killing effect of ozone on *L. monocytogenes* agar plates

Treatment	2 min		5 min		10 min		15 min	
Location in chamber	*Q1*	*Q2*	*Q1*	*Q2*	*Q1*	*Q2*	*Q1*	*Q2*
Survivor bacteria cfu/plate	TM*	TM	TM	2.12×10^3	2.57×10^3	8.96×10^2	1.79×10^3	1.05×10^3
Log reduction cfu/plate	-	-	-	2.45	2.37	2.82	2.52	2.75
Location in chamber	*Q3*	*Q4*	*Q3*	*Q4*	*Q3*	*Q4*	*Q3*	*Q4*
Survivor bacteria cfu/plate	TM	TM	TM	2.29×10^3	1.16×10^3	7.46×10^2	2.44×10^3	9.96×10^2
Log reduction cfu/plate	-	-	-	2.42	2.71	2.90	2.39	2.78
Control: 6.00×10^5 (cfu/plate)								

*TM: Too many to be counted

Table 3-43. Killing effect of ozone on *E. coli (lux)* on agar plates

Treatment	2 min		5 min		10 min		15 min	
Location in chamber	*Q1*	*Q2*	*Q1*	*Q2*	*Q1*	*Q2*	*Q1*	*Q2*
Survivor bacteria cfu/plate	TM	TM	1.08×10^3	6.50×10^2	5.83×10^2	4.32×10^2	1.97×10^2	1.12×10^2
Log reduction cfu/plate	-	-	2.57	2.79	2.83	2.96	3.31	3.55
Location in chamber	*Q3*	*Q4*	*Q3*	*Q4*	*Q3*	*Q4*	*Q3*	*Q4*
Survivor bacteria cfu/plate	TM	5.06×10^2	9.29×10^2	8.71×10^2	5.70×10^2	4.54×10^2	9.4×10^1	4.09×10^2
Log reduction cfu/plate	-	2.90	2.63	2.66	2.84	2.94	2.63	2.99
Control: 4.00×10^5 (cfu/plate)								

*TM: Too many to be counted

Table 3-44. Killing effect of ozone on *S. aureus* on agar plates

Treatment	2 min		5 min		10 min		15 min	
Location in chamber	*Q1*	*Q2*	*Q1*	*Q2*	*Q1*	*Q2*	*Q1*	*Q2*
Survivor bacteria cfu/plate	TM	TM	TM	1.64×10^3	2.05×10^3	1.54×10^2	4.02×10^2	2.04×10^2
Log reduction cfu/plate	-	-	-	2.39	2.29	3.41	3.00	3.29
Location in chamber	*Q3*	*Q4*	*Q3*	*Q4*	*Q3*	*Q4*	*Q3*	*Q4*
Survivor bacteria cfu/plate	TM	TM	1.43×10^4	8.97×10^2	4.83×10^2	3.06×10^2	1.91×10^2	2.52×10^2
Log reduction cfu/plate	-	-	2.44	2.65	2.92	3.11	3.32	3.20
Control: 4.00×10^5 (cfu/plate)								

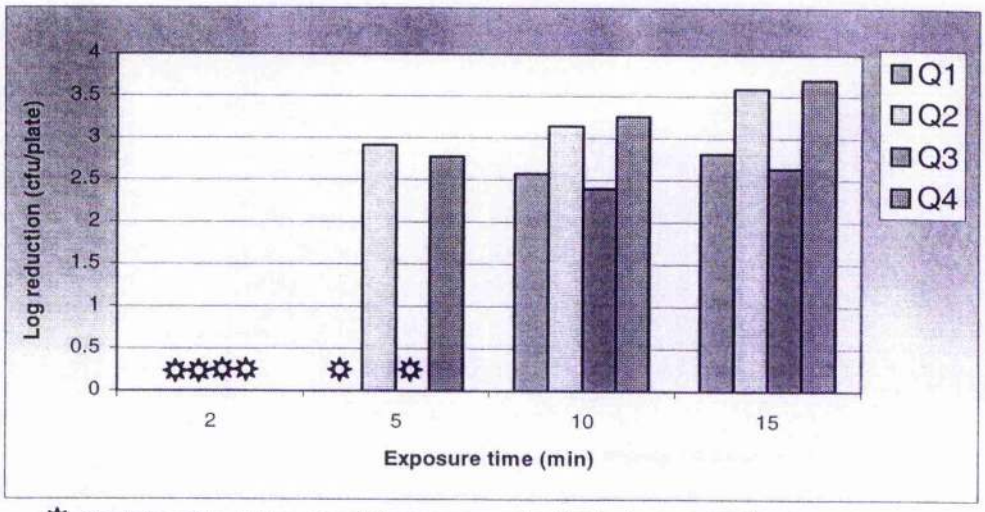
*TM: Too many to be counted

Table 3-45. Killing effect of ozone on *C. jejuni* on agar plates

Treatment	5 min		10 min		15 min	
Location in chamber	***Q1***	*Q2*	*Q1*	*Q2*	*Q1*	*Q2*
Survivor bacteria cfu/plate	**TM**	**TM**	**TM**	**TM**	**5.9×10^2**	**1.0×10^1**
Log reduction cfu/plate	-	-	-	-	**1.88**	**2.64**
Location in chamber	*Q3*	*Q4*	*Q3*	*Q4*	*Q3*	*Q4*
Survivor bacteria cfu/plate	**TM**	**TM**	**TM**	**TM**	**2.3×10^2**	**4.7×10^1**
Log reduction cfu/plate	-	-	-	-	**2.29**	**2.98**
Control: 4.50×10^4 (cfu/plate)**						

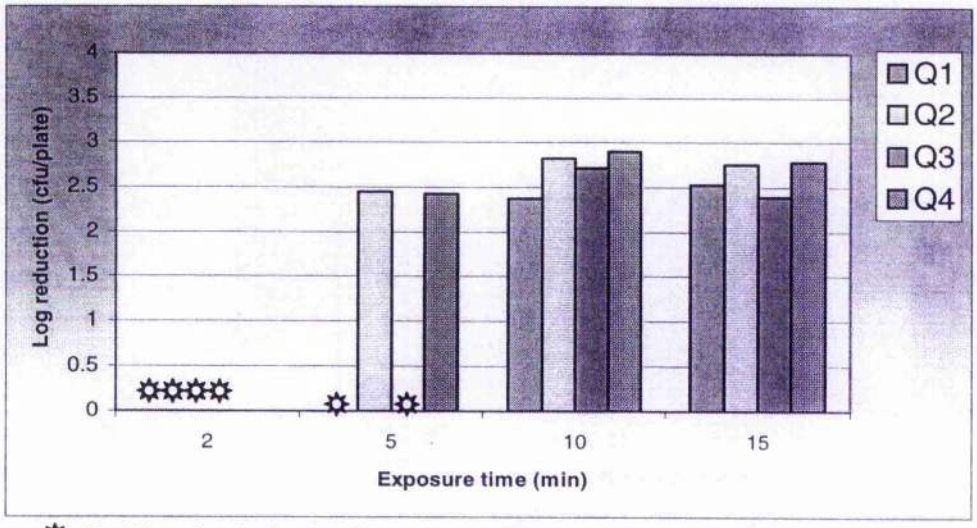
*TM: Too many to be counted

** Mean of recovered cfu after incubation under microaerophilic conditions



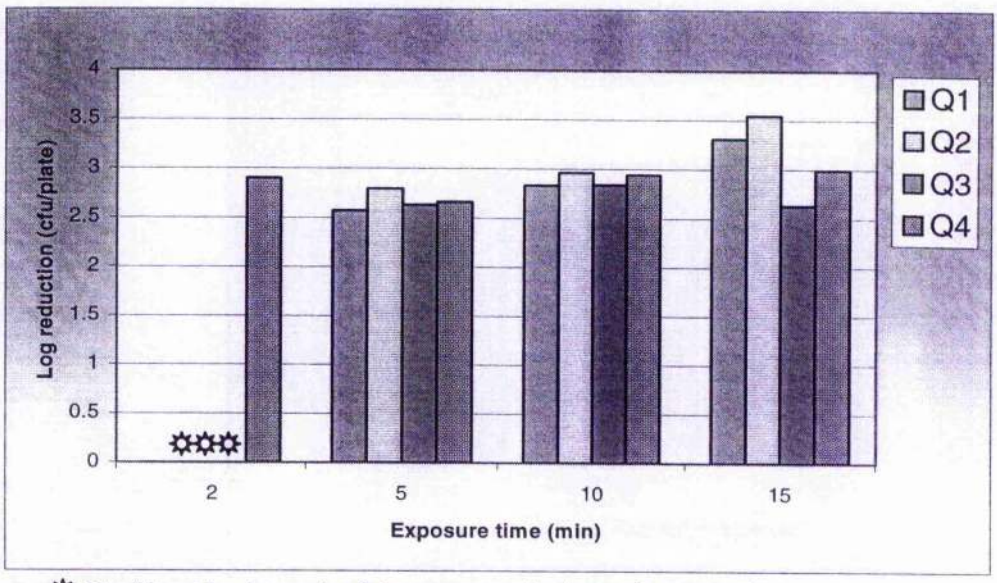
✡ Could not be determined (too many colonies to be counted)

Figure 3-13. Comparison of killing effect of different exposure times of ozonation of *S. typhimurium* on agar plates



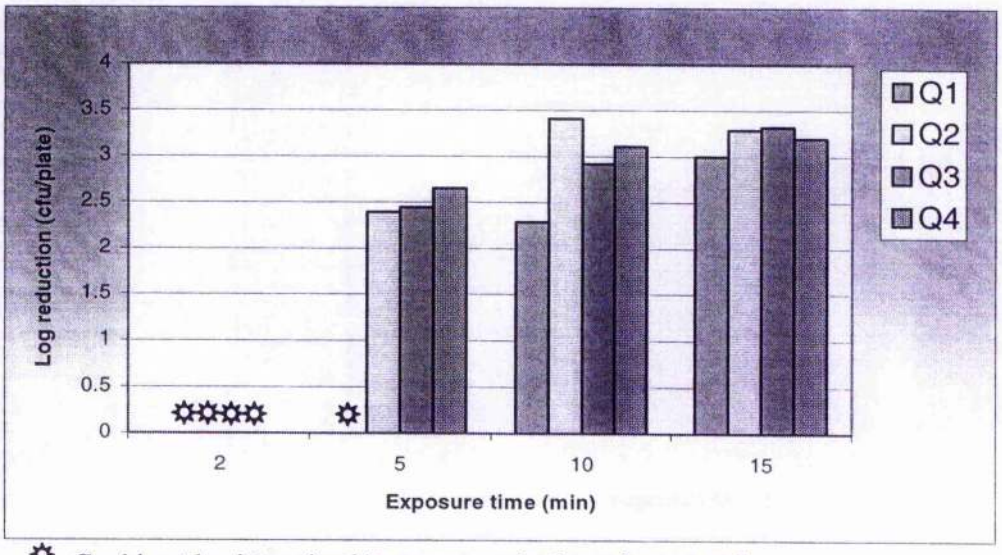
✡ Could not be determined (too many colonies to be counted)

Figure 3-14. Comparison of killing effect of different exposure times of ozonation of *L. monocytogenes* on agar plates



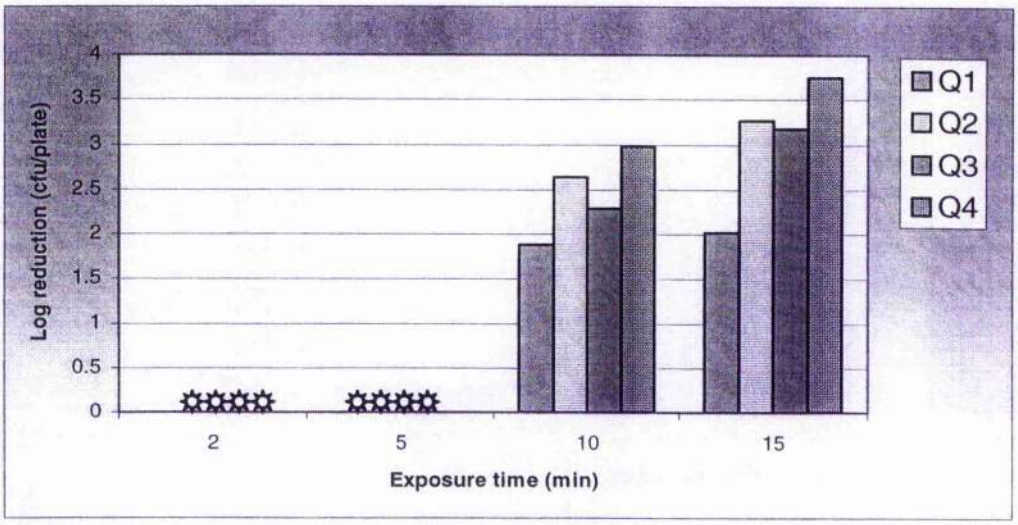
✡ Could not be determined (too many colonies to be counted)

Figure 3-15. Comparison of killing effect of different exposure times of ozonation of *E. coli (lux)* on agar plates



✡ Could not be determined (too many colonies to be counted

Figure 3-16. Comparison of killing effect of different exposure times of ozonation of *S. aureus* on agar plates



✡ Could not be determined (too many colonies to be counted)

Figure 3-17. Comparison of killing effect of different exposure time ozonation of *C. jejuni* on agar plates

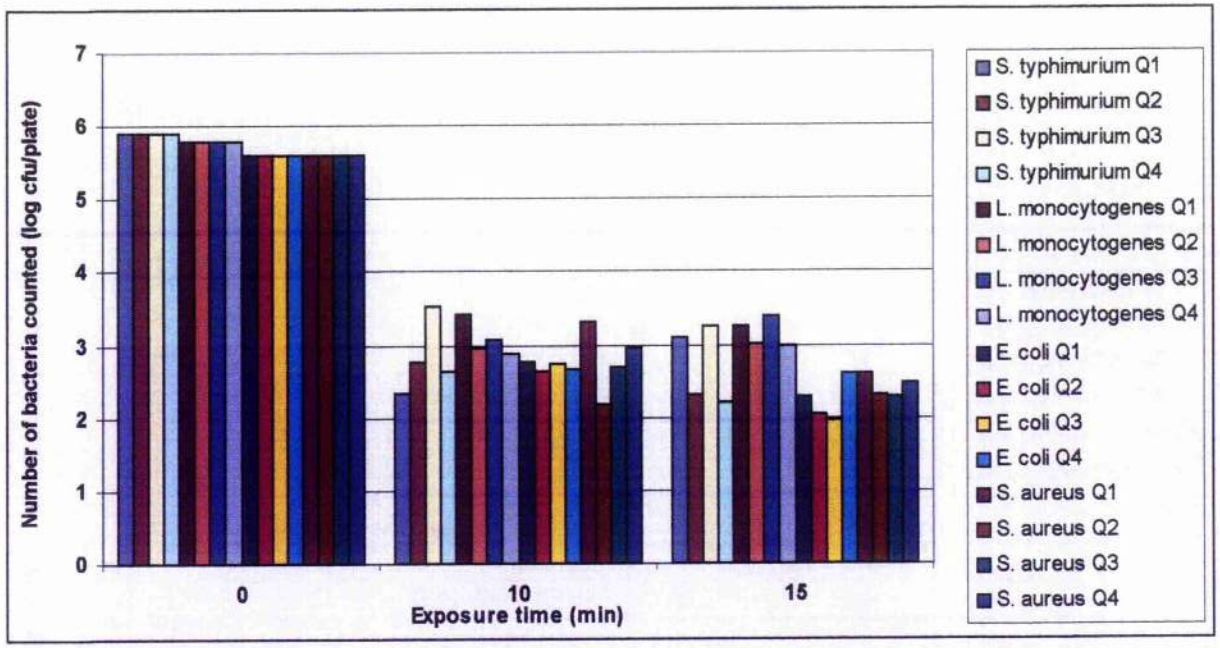


Figure 3-18. Bar graph showing cfu counts of *S. typhimurium, L. monocytogenes, S. aureus and E. coli (lux)* inoculated on agar plates after different period of ozonation

Figure 3-19. Distribution of colonies of *S. typhimurium* after ozonation for 2 min

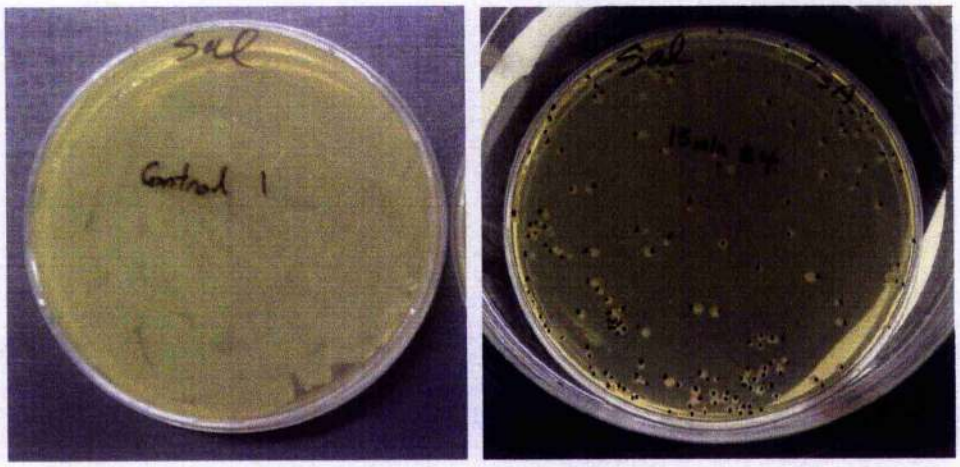


Figure 3-20. Plates of *S. typhimurium*, Left: control showing confirmation of growth, Right: after ozonation for 15 min

3.7 Investigation of bactericidal effects of high-power Nd:YAG and CO_2 laser radiation on selected bacteria on lawned agar

This experiment was done to investigate the killing effects of two kinds of laser radiation on bacteria on solid surfaces. In previous experiments, the killing effect of different treatments, including Nd:YAG laser, in liquid substrates was studied. The current study on agar is more relevant to the killing of bacteria on solid surfaces such as fish or other foods. In these experiments the different parameters, pulse repetition frequency (Nd:YAG laser and CO_2 laser) or continuous wave (CO_2 laser), power output, and different exposure times were studied. *E. coli (lux), S. putrefaciens, P. fragi, M. luteus* and *L. monocytogenes* were the target organisms.

3.7.1 Nd:YAG laser

Two pulse energies, 8 and 24 J, delivered over 8 ms were used and the frequency was varied between 5 and 30 Hz. The exposure time was adjusted from the 5 to 48 s. **Tables 3-46** shows 4 sets of parameters, with different exposure times to give different energy densities (calculation based on section 2.2.2) for treatment of 4 bacterial strains. The calculated beam area was about 1.5 cm^2. **Figure 3-21** shows the effect of the different parameters on *M. luteus*. Although differences in killing between the different sets of parameters were small, the higher frequency (f =30) always gave a greater zone of killing than the lower frequency (f =5). The energy density required to make a clear area equal to the laser beam area, for all sets of parameters, was about 2900 J/cm^2. Results for *E. coli (lux)* are shown in **Figure 3-22.** Again, the higher frequency gave a greater clear area than the lower frequency. This bacterium was slightly more sensitive to laser radiation than *M luteus* in that an energy density of 2700 J/cm^2 was required to produce a clear area equal to the beam area for most sets of parameters. As shown in **Figure 3-23** for *P. fragi* and in **Figure 3-24** for *S. putrefaciens,* although no large differences were seen between the various sets of parameters for killing of the bacteria, the higher frequencies, again, gave a greater a zone of killing. The energy densities for making the clear area equal to the laser beam area were about 2300 and 1900 J/cm^2 for *P. fragi* and *S. putrefaciens*, respectively. Thus, *M. luteus*, as a Gram-positive bacterium proved to be the most resistant bacterium and *S. putrefaciens* the most sensitive bacterium.

Table 3-46. Nd:YAG laser parameters used for treatment of bacteria on agar

	Power (W)	Frequency (Hz)	Time (S)	Calculated energy density (J/cm^2)
Parameter set 1	24	5	10	554
	24	5	12	665
	24	5	14	775
	24	5	16	886
	24	5	18	997
	24	5	20	1108
	24	5	24	1330
	24	5	36	1995
	24	5	48	2660
Parameter set 2	24	10	5	577
	24	10	6	692
	24	10	7	807
	24	10	8	923
	24	10	9	1038
	24	10	10	1154
	24	10	12	1384
	24	10	18	2077
	24	10	24	2769
Parameter set 3	8	15	10	554
	8	15	12	665
	8	15	14	775
	8	15	16	886
	8	15	18	997
	8	15	20	1108
	8	15	24	1330
	8	15	36	1995
	8	15	48	2660
Parameter set 4	8	30	5	577
	8	30	6	692
	8	30	7	807
	8	30	8	923
	8	30	9	1038
	8	30	10	1154
	8	30	12	1384
	8	30	18	2077
	8	30	24	2769

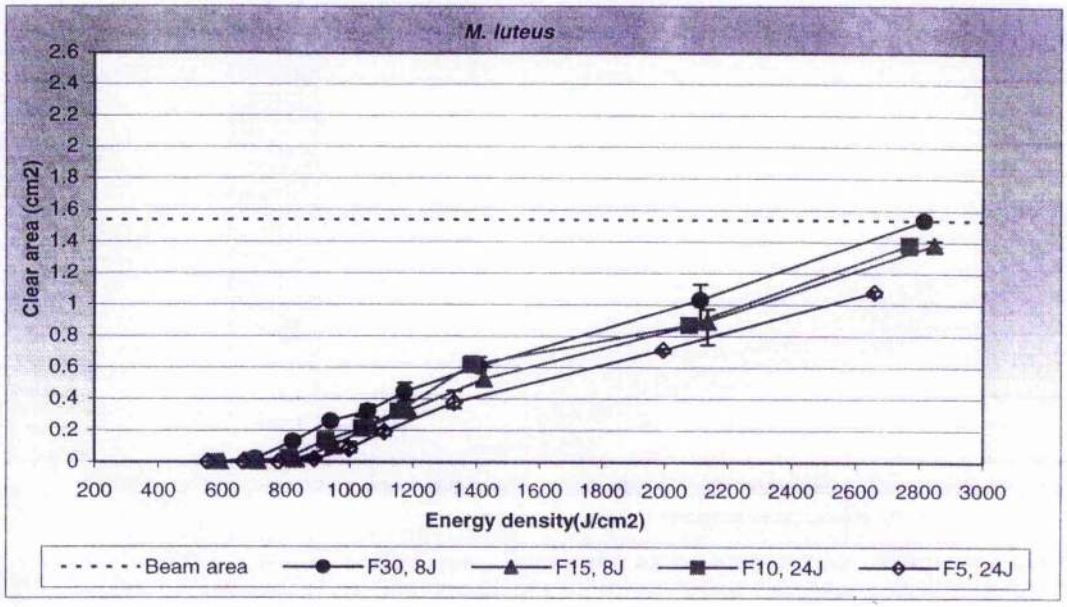


Figure 3-21. Killing effect of different pulse frequencies and power output by Nd:YAG laser on *M. luteus* on agar plates

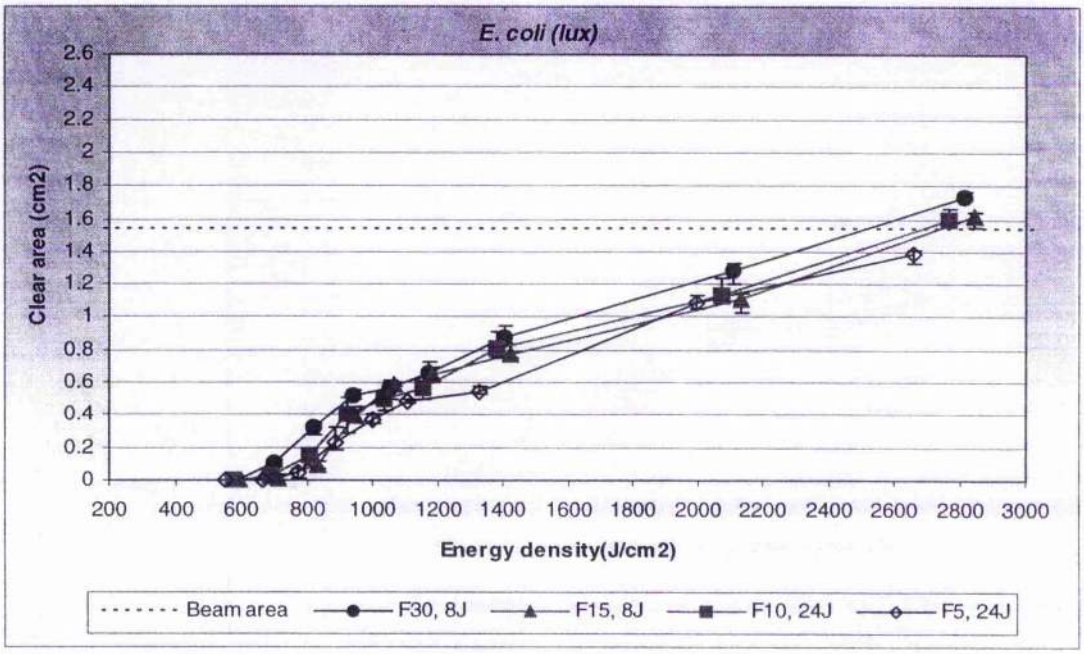


Figure 3-22. Killing effect of different pulse frequencies and power output by Nd:YAG laser on *E. coli (lux)* on agar plates

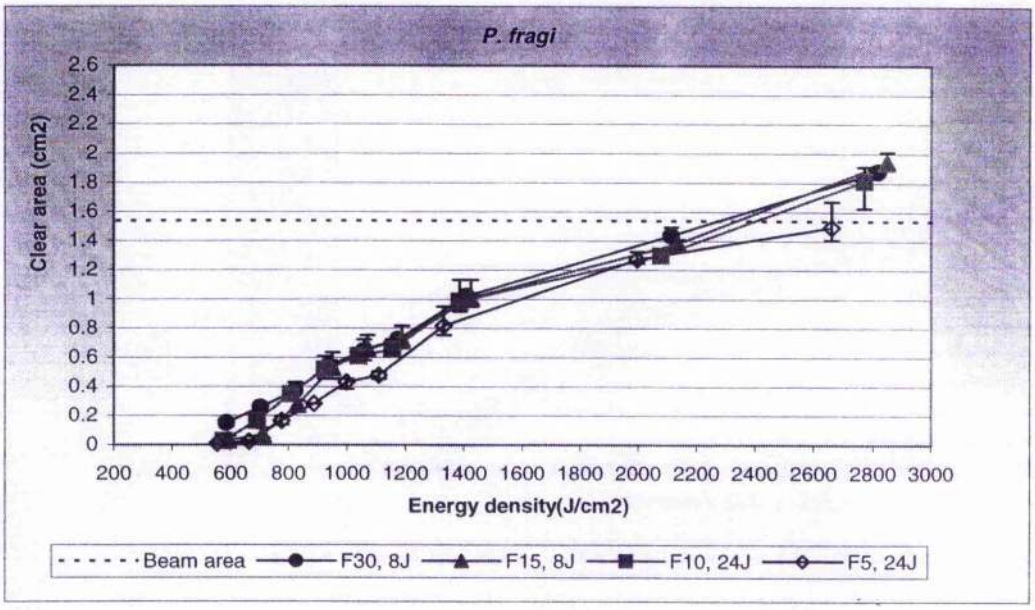


Figure 3-23. Killing effect of different pulse frequencies and power output by Nd:YAG laser on *P. fragi* on agar plates

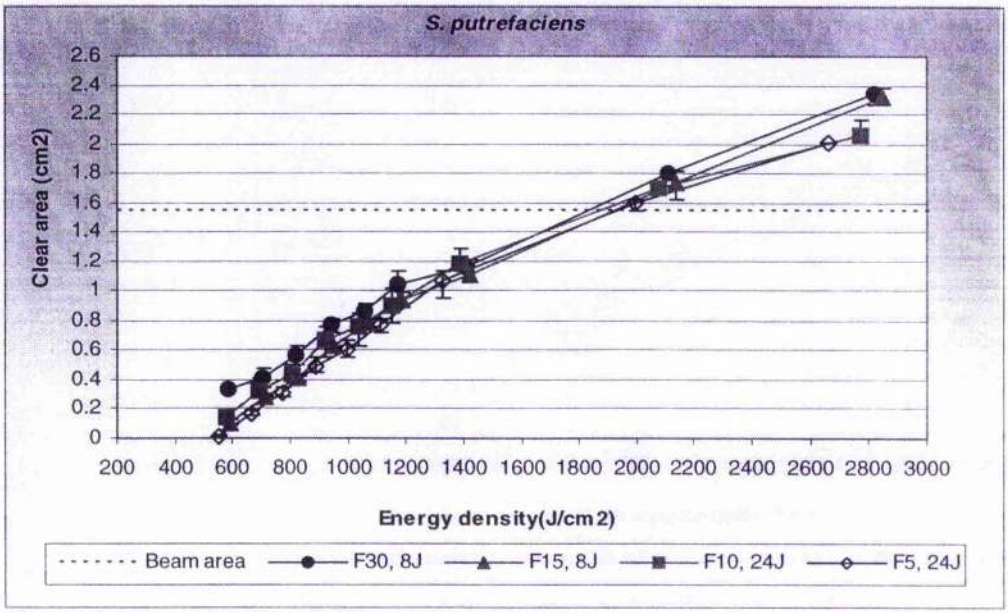


Figure 3-24. Killing effect of different pulse frequencies and power output by Nd:YAG laser on *S. putrefaciens* on agar plates

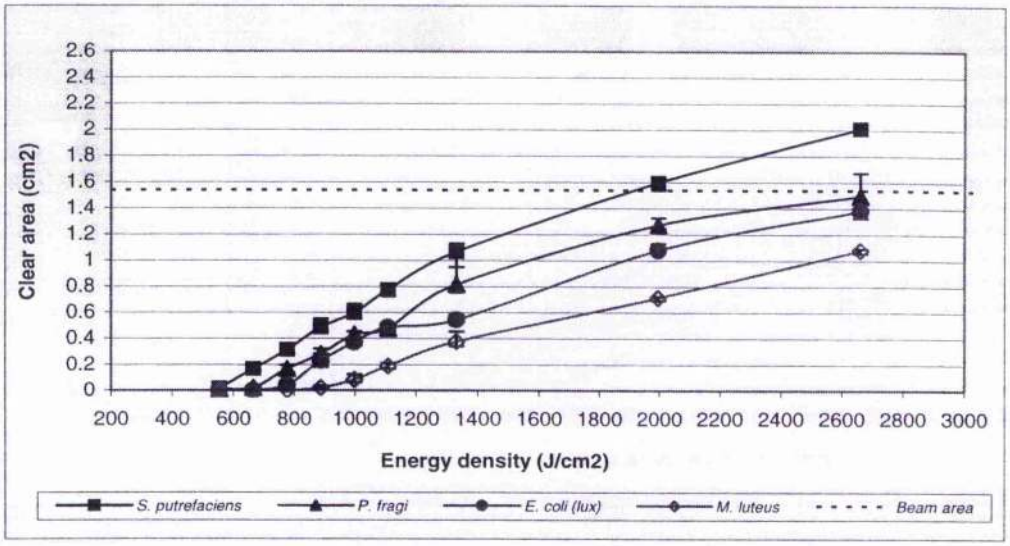


Figure 3-25. Comparison of sensitivity of bacteria on agar plate to Nd:YAG laser irradiation (pulse energy 24J, frequency 5 Hz)

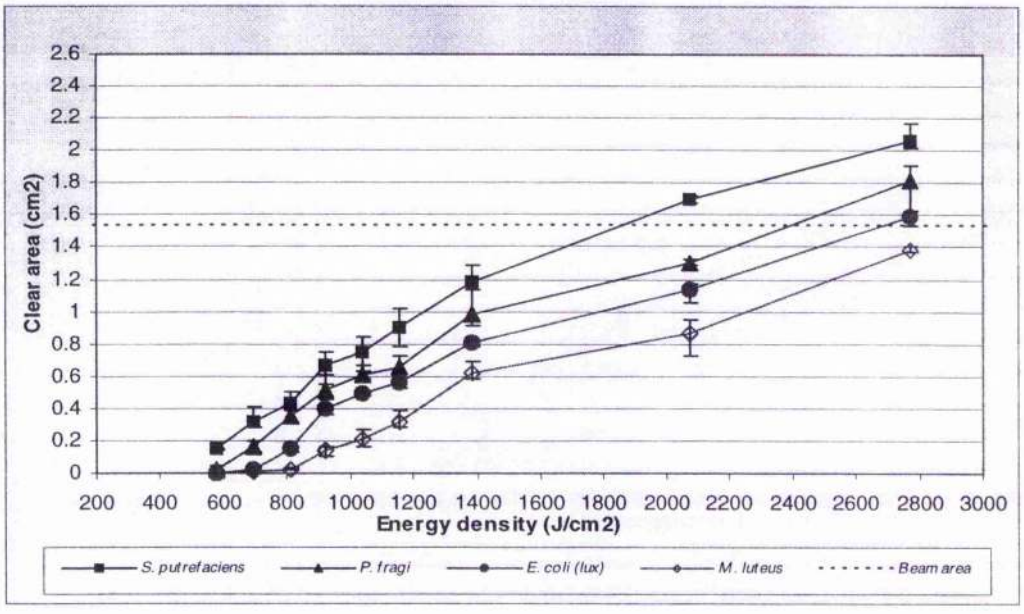


Figure 3-26. Comparison of sensitivity of bacteria on agar plate to Nd:YAG laser irradiation (pulse energy 24J, frequency 10 Hz)

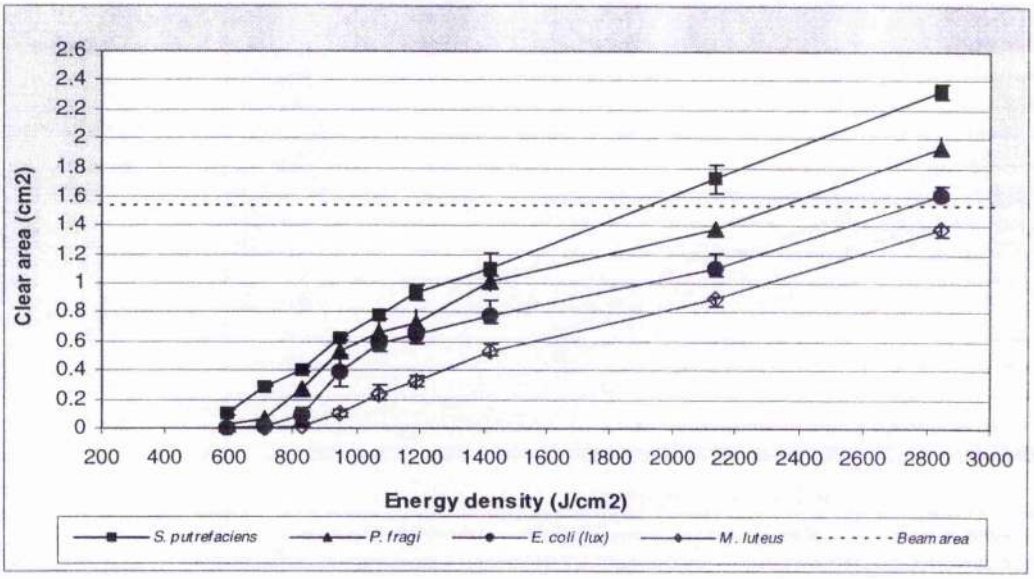


Figure 3-27. Comparison of sensitivity of bacteria on agar plate to Nd:YAG laser irradiation (pulse energy 8J, frequency 15 Hz)

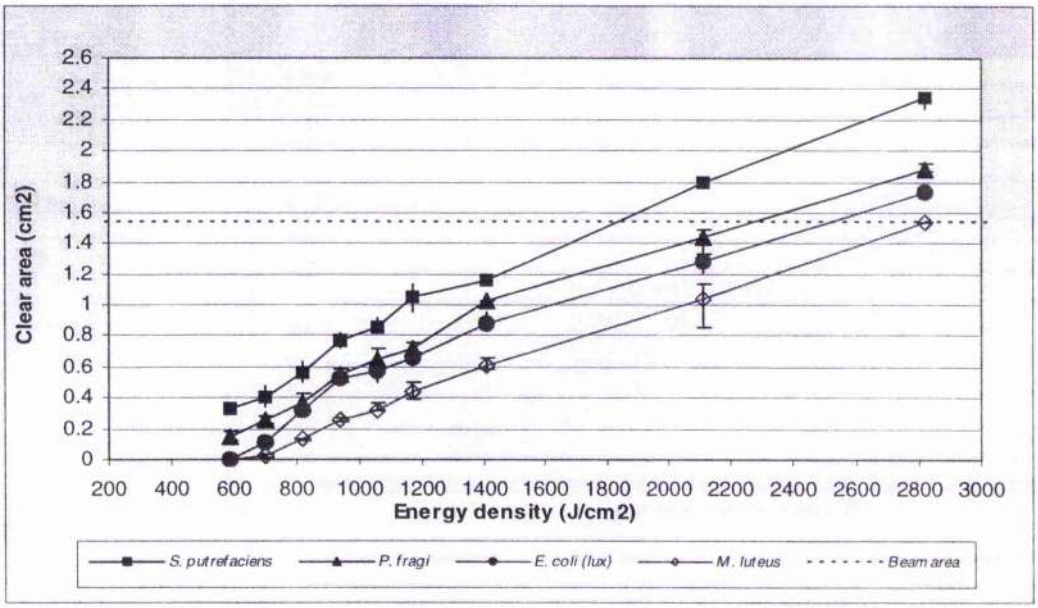


Figure 3-28. Comparison of sensitivity of bacteria on agar plate to Nd:YAG laser irradiation (pulse energy 8J, frequency 30 Hz)

Figures 3-25, 3-26, 3-27 and 3-28 show comparisons of the sensitivity of the different bacteria on agar plates to killing by the different laser treatments. Again these comparisons showed that *S. putrefaciens* was the most sensitive bacterium to the treatments followed by *P. fragi*, *E. coli (lux)* and *M. luteus*. For example, the energy densities equal to 1400 J/cm^2 (with different settings) gave about 0.6 cm^2 greater clear area with *S. putrefaciens* than with *M. luteus*. The difference between *M. luteus* and *P. fragi* was 0.4 cm^2 and between *M. luteus* and *E. coli* was 0.3 cm^2.

3.7.2 CO_2 laser

In this experiment, two power outputs (100 W and 200 W) were chosen and different frequencies from 5, 10, 20 and 50 Hz to continuous wave, were investigated for their killing effect on *M. luteus*, *P. fragi*, *E. coli (lux)* and *S. putrefaciens* on agar plates (**Figure 3-29**).

The killing effect of the CO_2 laser at 100 W with various frequencies: 5, 10, 20 and 50 Hz, are shown in **Figures 3-30, 3-31, 3-32 and 3-33**. At low frequencies, 5 and 10 Hz, *P. fragi* was the most sensitive bacterium and *M. luteus* was the most resistant (**Figures 3-30, 3-31**). At higher frequencies, however, 20 and 50 Hz, *E. coli (lux)* was the most sensitive bacterium (**Figures 3-32, 3-33**). Also at higher frequencies, the differences between the clear areas for different bacteria were more apparent. Whereas at frequencies of 5 and 10 Hz the difference between the clear areas for the most sensitive and resistant bacterium was about 0.2 cm^2, at higher frequencies the value was about 0.4 cm^2. This difference was not observed for the laser operating at 200 W with different frequencies, where the results are shown in **Figures 3-34, 3-35, 3-36 and 3-37**. With these parameters, it was observed that *P. fragi* was the most sensitive bacterium followed by *S. putrefaciens*, *E. coli* and finally *M. luteus*. Also, by using the laser at 200 W, the differences between the clear areas for the most sensitive and resistant bacterium was about 0.2 cm^2.

Figures 3-38 to 3-45 show the killing effect of the CO_2 laser at 100 and 200 W, delivered by continuous wave or at 4 different frequencies (5, 10, 20 and 50 Hz) on each bacterial strain on agar plates. For each organism, no significant differences were

observed for the various frequencies, whereas the differences between continuous wave and the laser's pulse mode were significant for all strains and at both power settings. Energy densities delivered by the continuous wave mode always gave clear areas greater by 0.5 to 0.7 cm^2, compared to those obtained with similar energy densities delivered by the pulse mode at different frequencies.

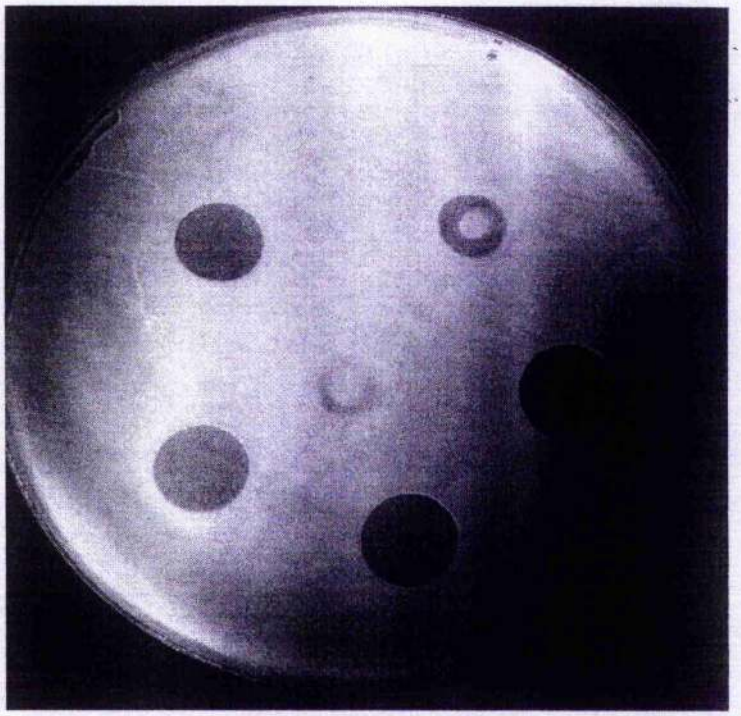


Figure 3-29. Killing effect of CO_2 laser on *M. luteus* on agar plate. Exposure to different energy densities made clear areas with no growth of the bacterium

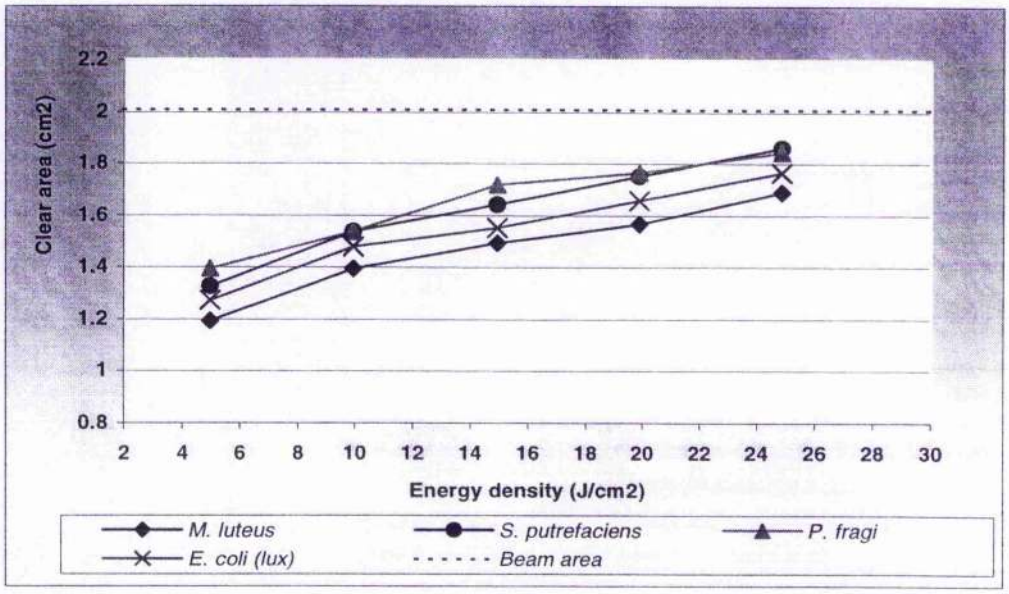


Figure 3-30. Killing effect of CO_2 laser (power output 100 W, frequency 5 Hz) on bacteria on agar plates

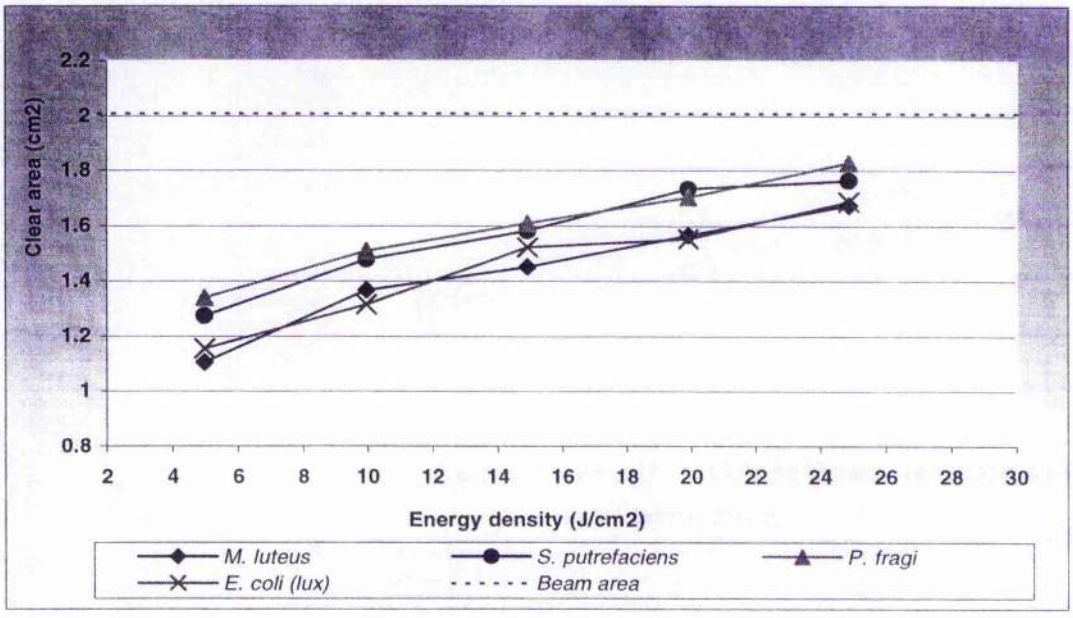


Figure 3-31. Killing effect of CO_2 laser (power output 100 W, frequency 10 Hz) on bacteria on agar plates

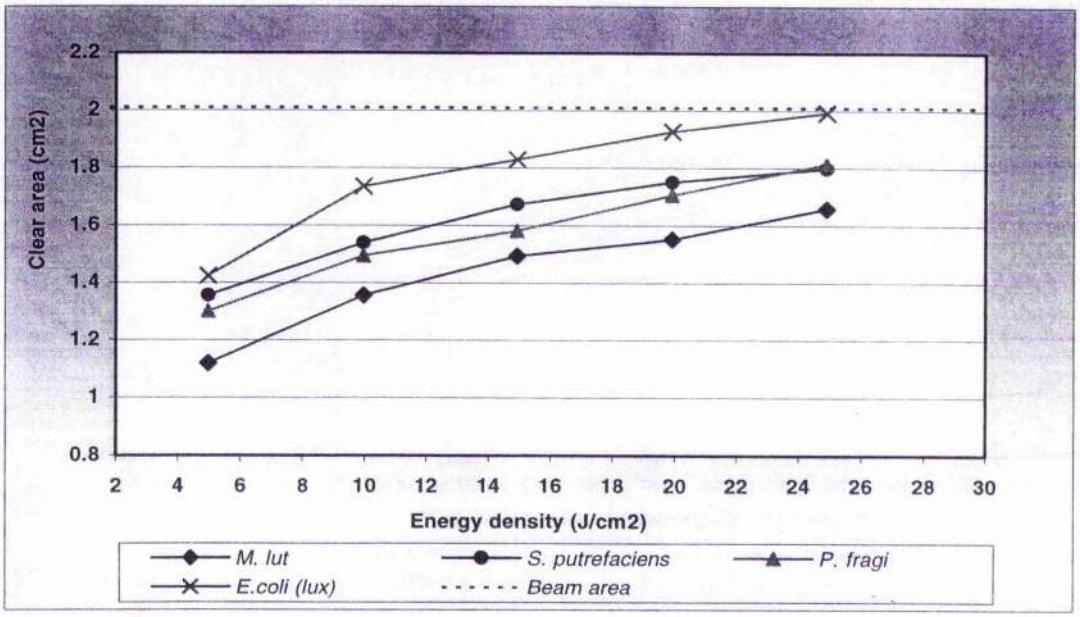


Figure 3-32. Killing effect of CO_2 laser (power output 100 W, frequency 20 Hz) on bacteria on agar plates

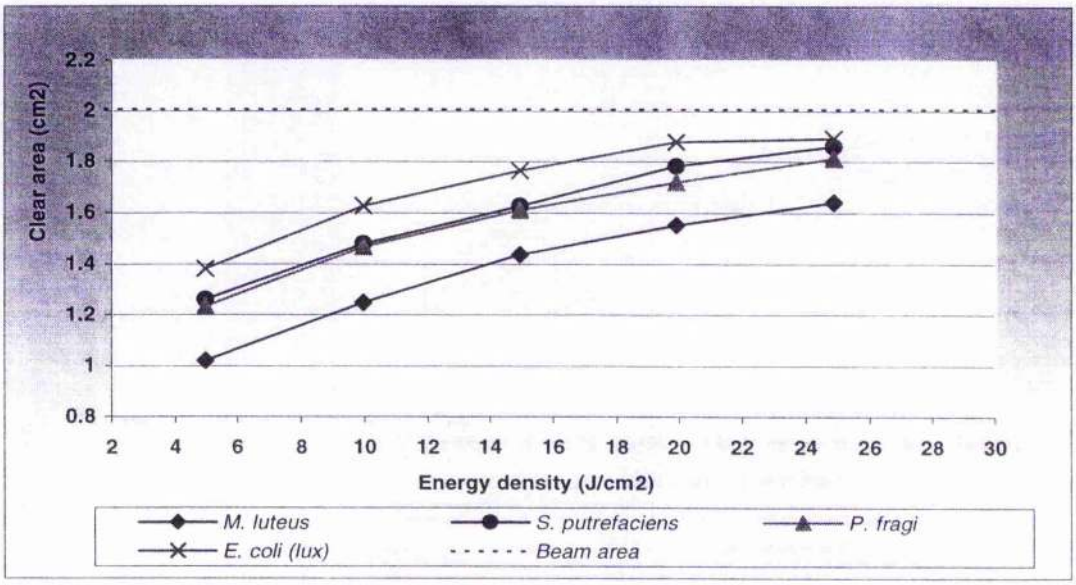


Figure 3-33. Killing effect of CO_2 laser (power output 100 W, frequency 50 Hz) on bacteria on agar plates

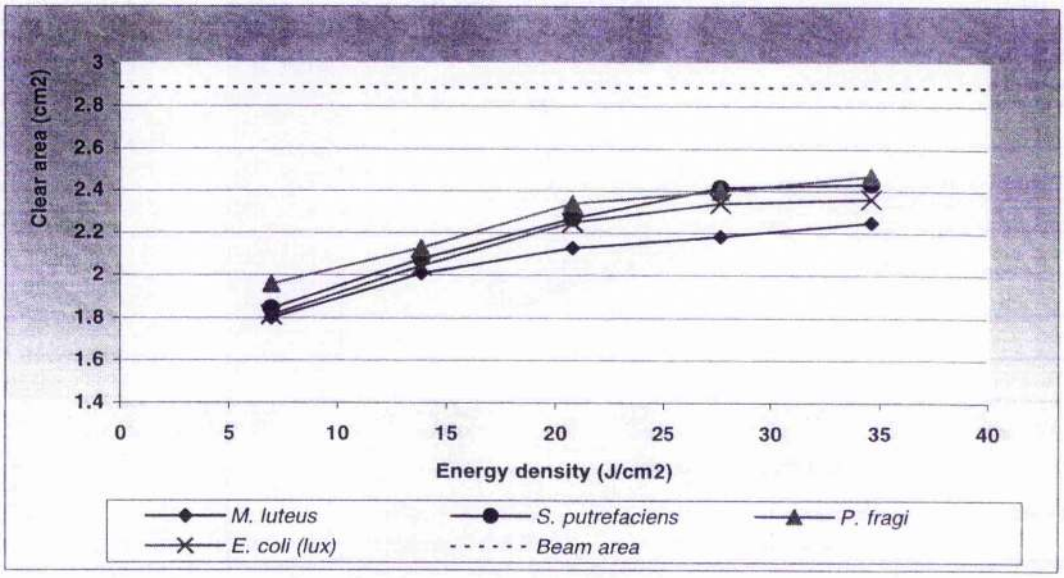


Figure 3-34. Killing effect of CO_2 laser (power output 200 W, frequency 5 Hz) on bacteria on agar plates

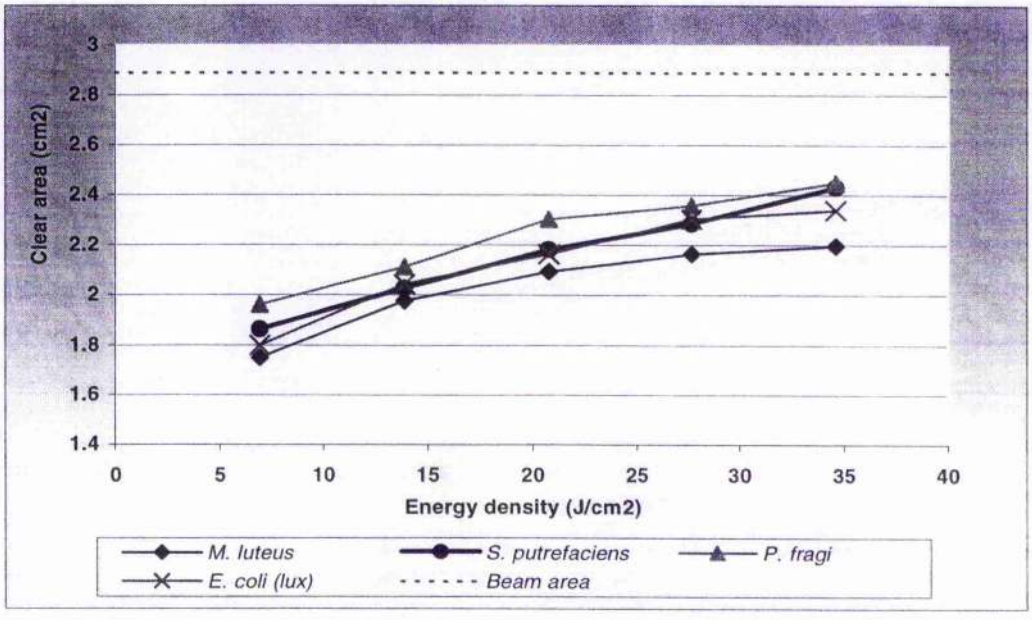


Figure 3-35. Killing effect of CO_2 laser (power output 200 W, frequency 10 Hz) on bacteria on agar plates

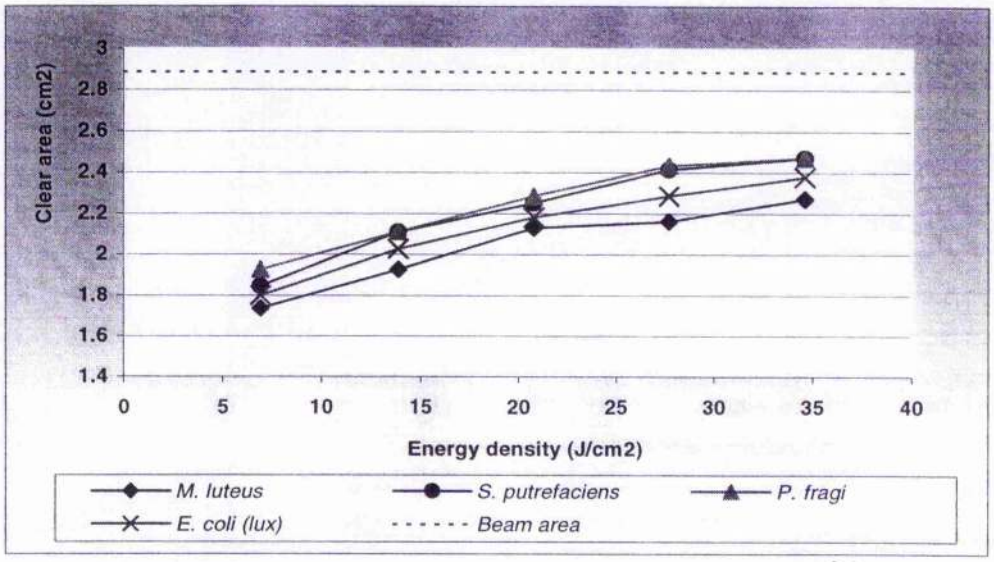


Figure 3-36. Killing effect of CO_2 laser (power output 200 W, frequency 20 Hz) on bacteria on agar plates

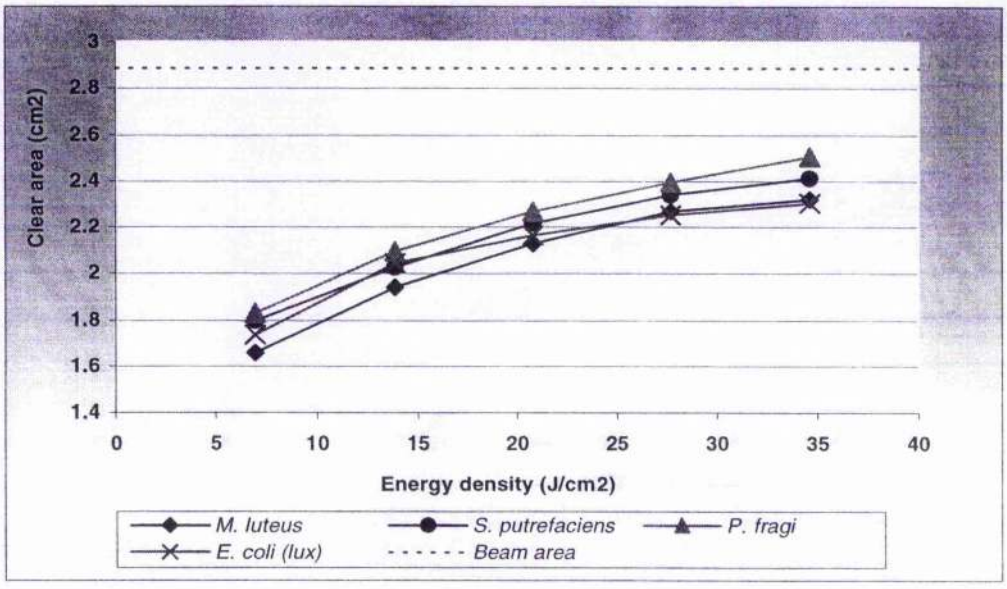


Figure 3-37. Killing effect of CO_2 laser (power output 200 W, frequency 50 Hz) on bacteria on agar plates

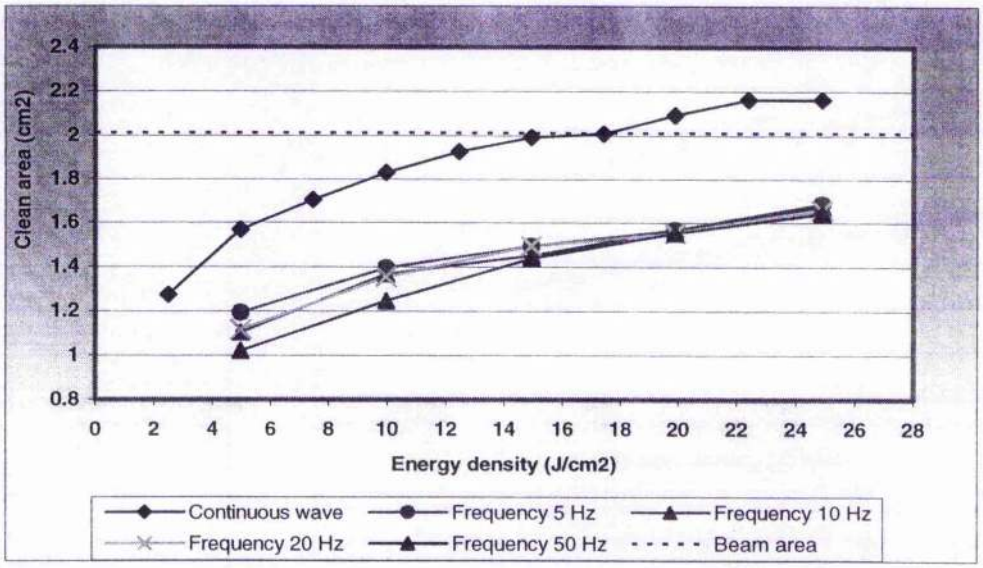


Figure 3-38. Comparison of killing effect of continuous wave and different frequencies of CO_2 laser (power output 100 W) on *M. luteus* on agar plates

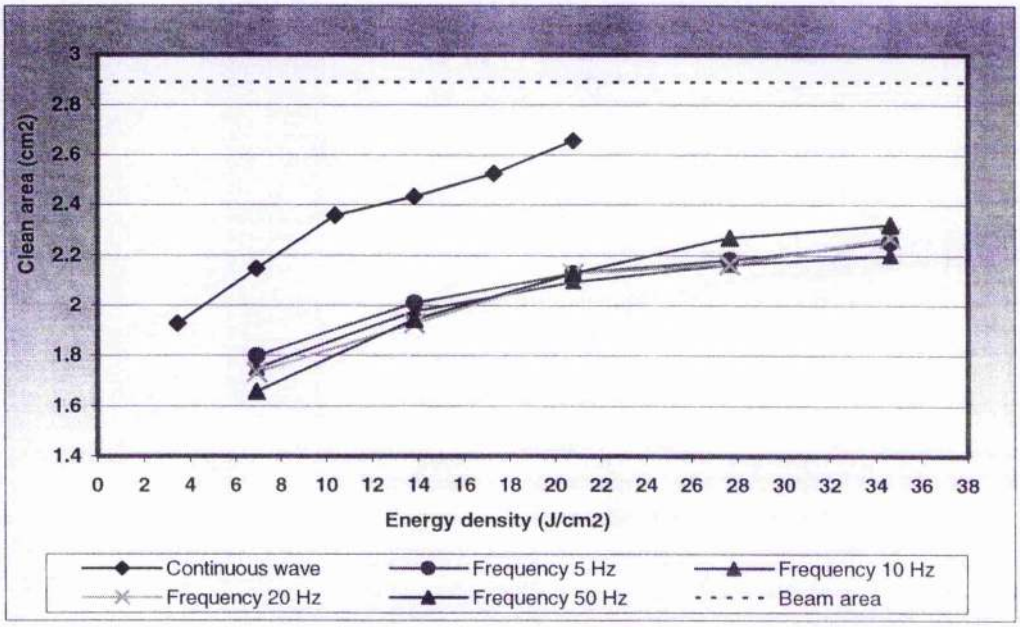


Figure 3-39. Comparison of killing effect of continuous wave and different frequencies of CO_2 laser (power output 200 W) on *M. luteus* on agar plates

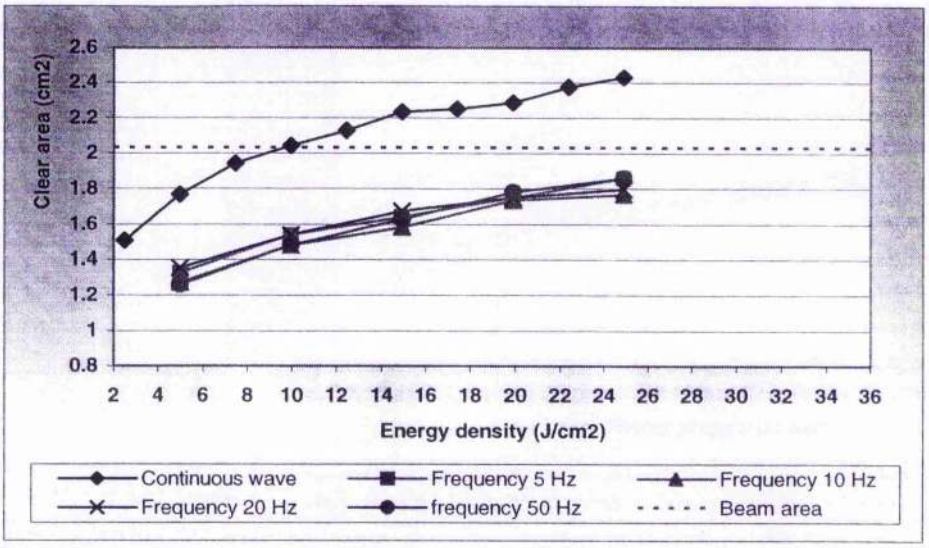


Figure 3-40. Comparison of killing effect of continuous wave and different frequencies of CO_2 laser (power output 100 W) on *S. putrefaciens* on agar plates

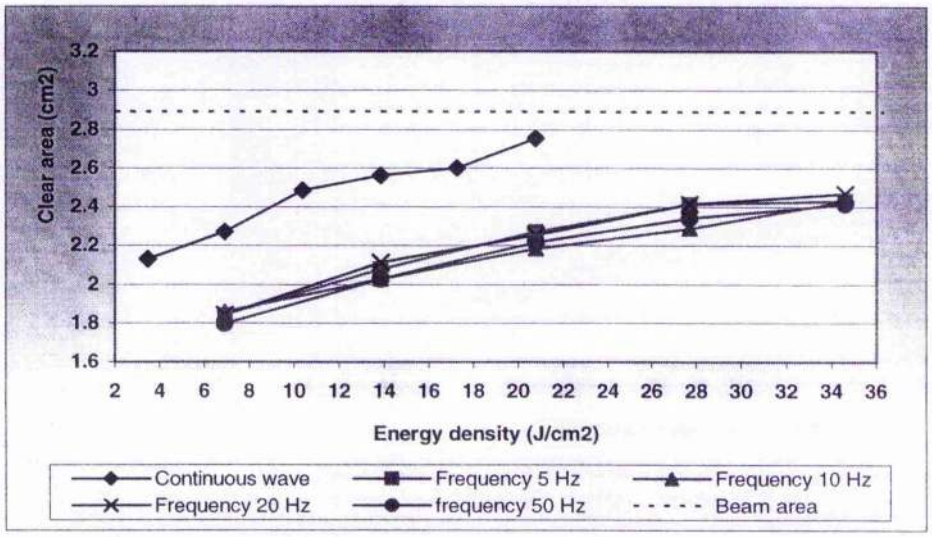


Figure 3-41. Comparison of killing effect of continuous wave and different frequencies of CO_2 laser (power output 200 W) on *S. putrefaciens* on agar plates

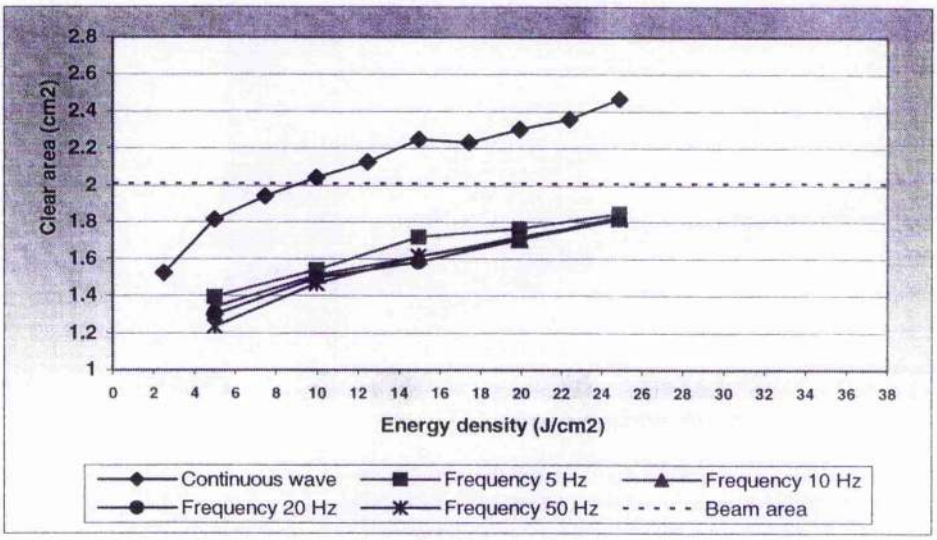


Figure 3-42. Comparison of killing effect of continuous wave and different frequencies of CO_2 laser (power output 100 W) on *P. fragi* on agar plates

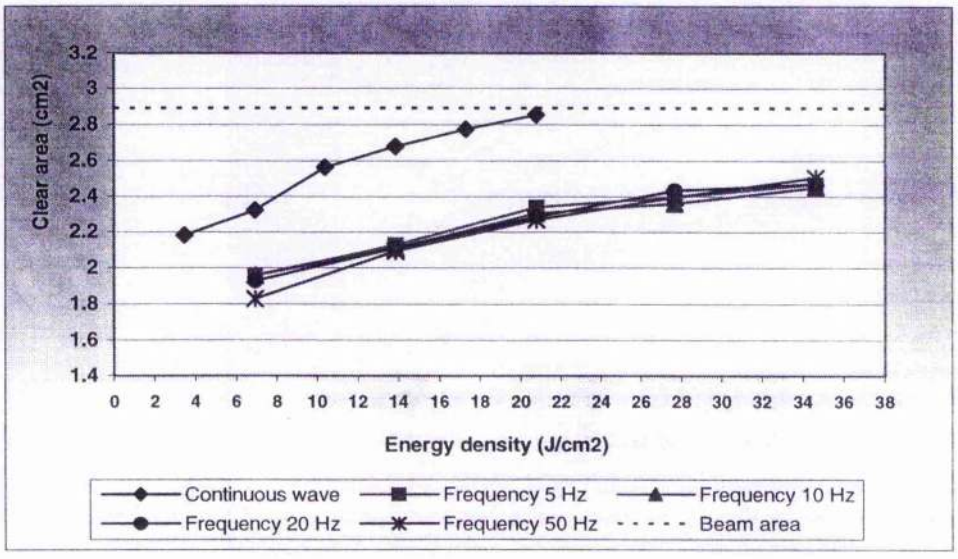


Figure 3-43. Comparison of killing effect of continuous wave and different frequencies of CO_2 laser (power output 200 W) on *P. fragi* on agar plates

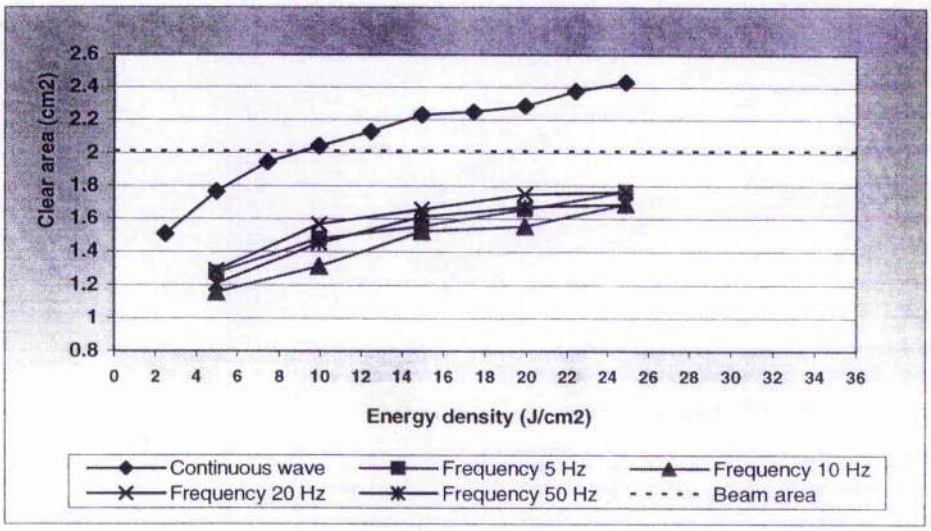


Figure 3-44. Comparison of killing effect of continuous wave and different frequencies of CO_2 laser (power output 100 W) on *E. coli (lux)* on agar plates

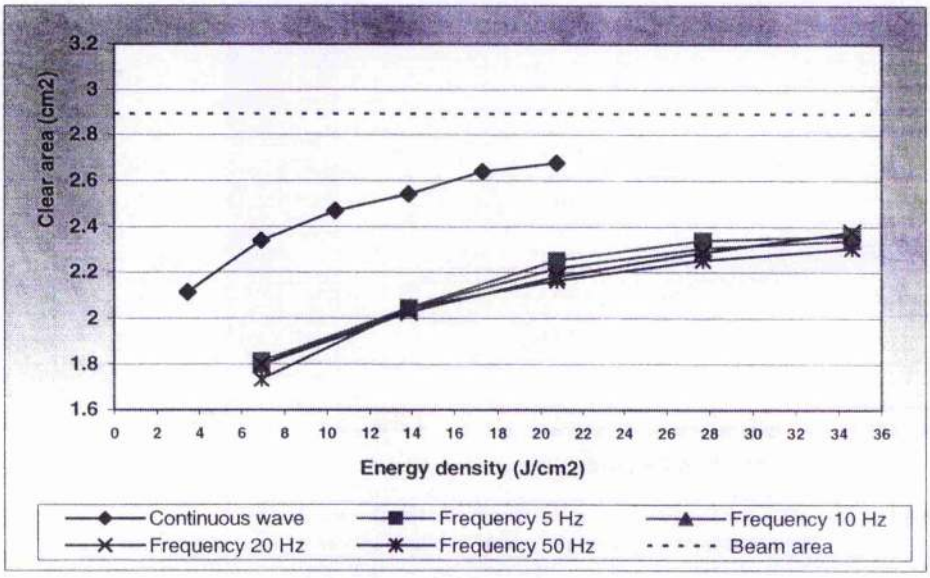


Figure 3-45. Comparison of killing effect of continuous wave and different frequencies of CO_2 laser (power output 200 W) on *E. coli (lux)* on agar plates

As observed in the above results, a continuous wave output always gave a greater zone of cleaning compared to the pulse mode. So, in subsequent experiments, the killing effect of continuous wave output at different powers (20, 50, 100 and 500 W) was studied with the bacteria on agar plates. *S. putrefaciens* as a sensitive bacterium and *M. luteus* as a resistant bacterium were chosen at the target organisms. Also *L. monocytogenes* was investigated and all results were compared. **Figure 3-46** shows the effect of the CO_2 laser radiation set at 20 W power on the three species. Although *L. monocytogenes* is a Gram-positive bacterium, its sensitivity to this CO_2 laser treatment on agar plates was very similar to that of the Gram-negative bacterium *S. putrefaciens* rather than to *M. luteus*. Similar results were apparent when higher powers were used. Results at 50, 100 and 500W are shown in **Figures 3-47, 3-48 and 3-49**. Melted agar was observed with 100W power and when the energy density was increased to 23 J/cm^2. With 500W power, melted agar was observed at 3.3 J/cm^2. In **Figures 3-50, 3-51 and 3-52** the effect of different power settings are compared for each bacterium. The clear area on agar was increased when the power was increased, where similar energy densities were used. It should be noticed, however, that the beam area increased with increasing power. When 500W of power was used, the largest clear areas were observed. For example 4 J/cm^2 with 50W power gave 0.3 cm^2 clear area for *M. luteus*, whereas the value for 100 W was 0.9 and for 500W was 1.9 cm^2. No clear area was observed for 20 W at the same energy density. *S. putrefaciens* was more sensitive to the treatment. At an energy density of 4 J/cm^2 with 20, 50, 100 and 500 W, clear areas of about 0.15, 0.5, 1.4 and 2.1 cm^2 were obtained respectively.

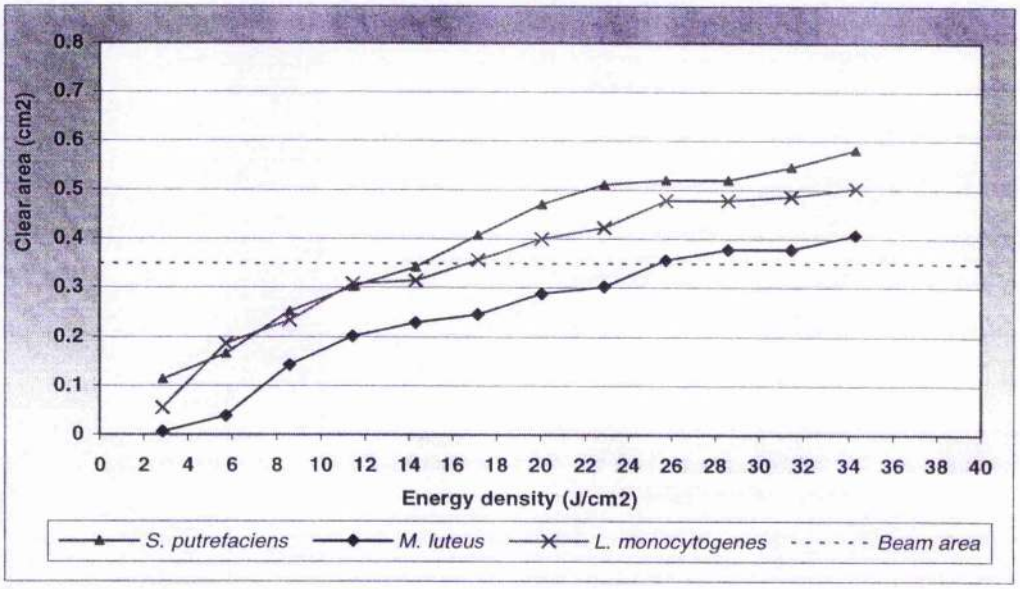


Figure 3-46. Comparison of killing effect of continuous wave CO_2 laser at power output 20 W on selected bacteria

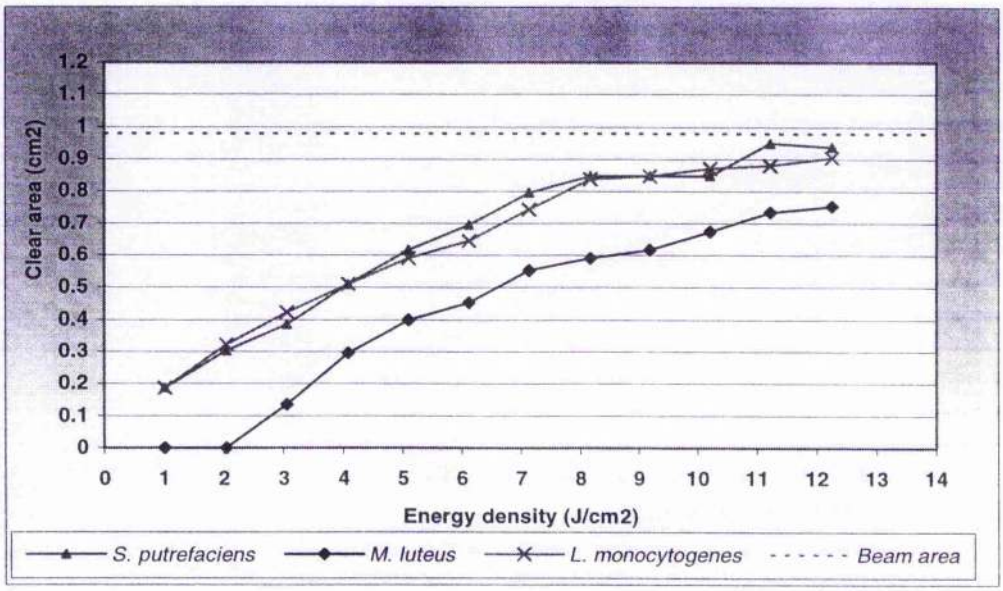


Figure 3-47. Comparison of killing effect of continuous wave CO_2 laser at power output 50 W on selected bacteria

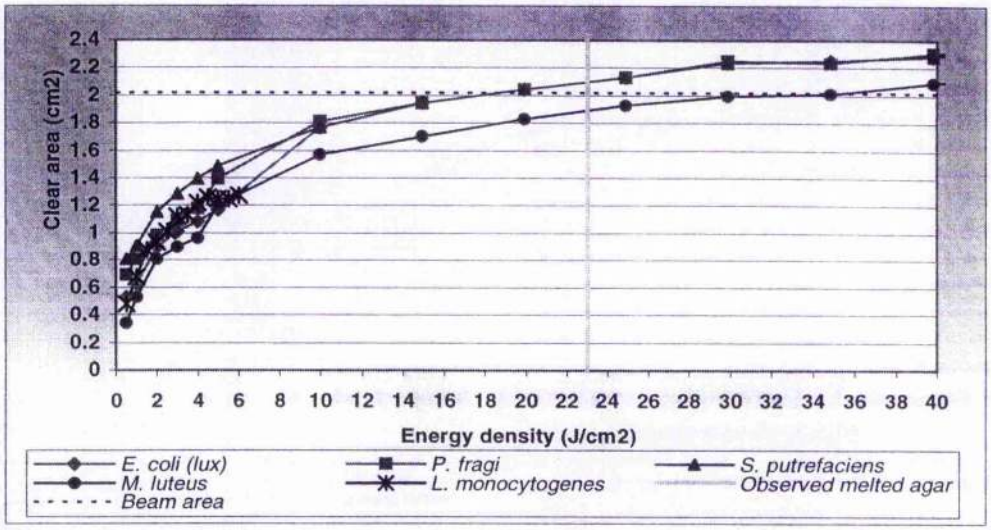


Figure 3-48. Comparison of killing effect of continuous wave CO_2 laser at power output 100 W on selected bacteria

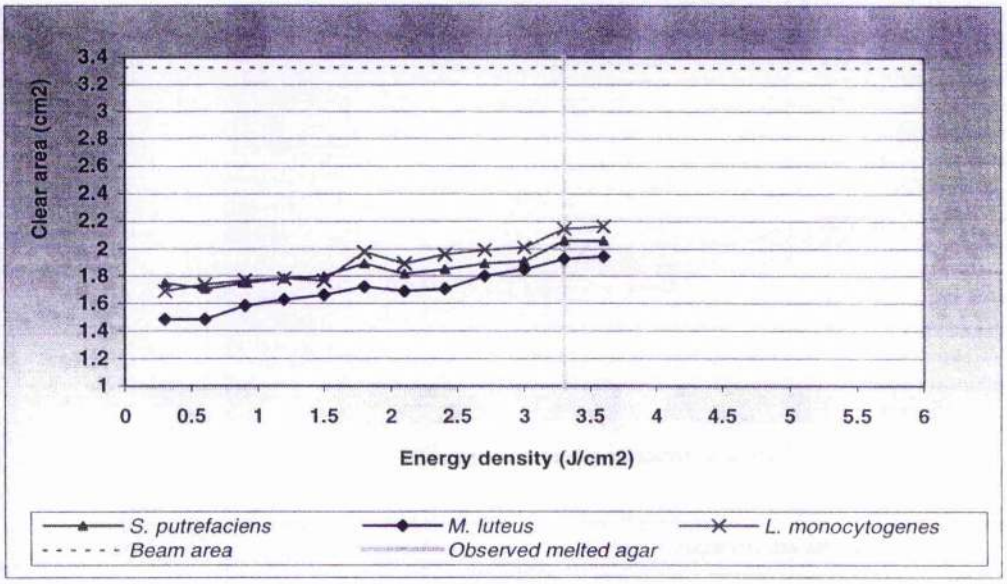


Figure 3-49. Comparison of killing effect of continuous wave CO_2 laser at power output 500 W on selected bacteria

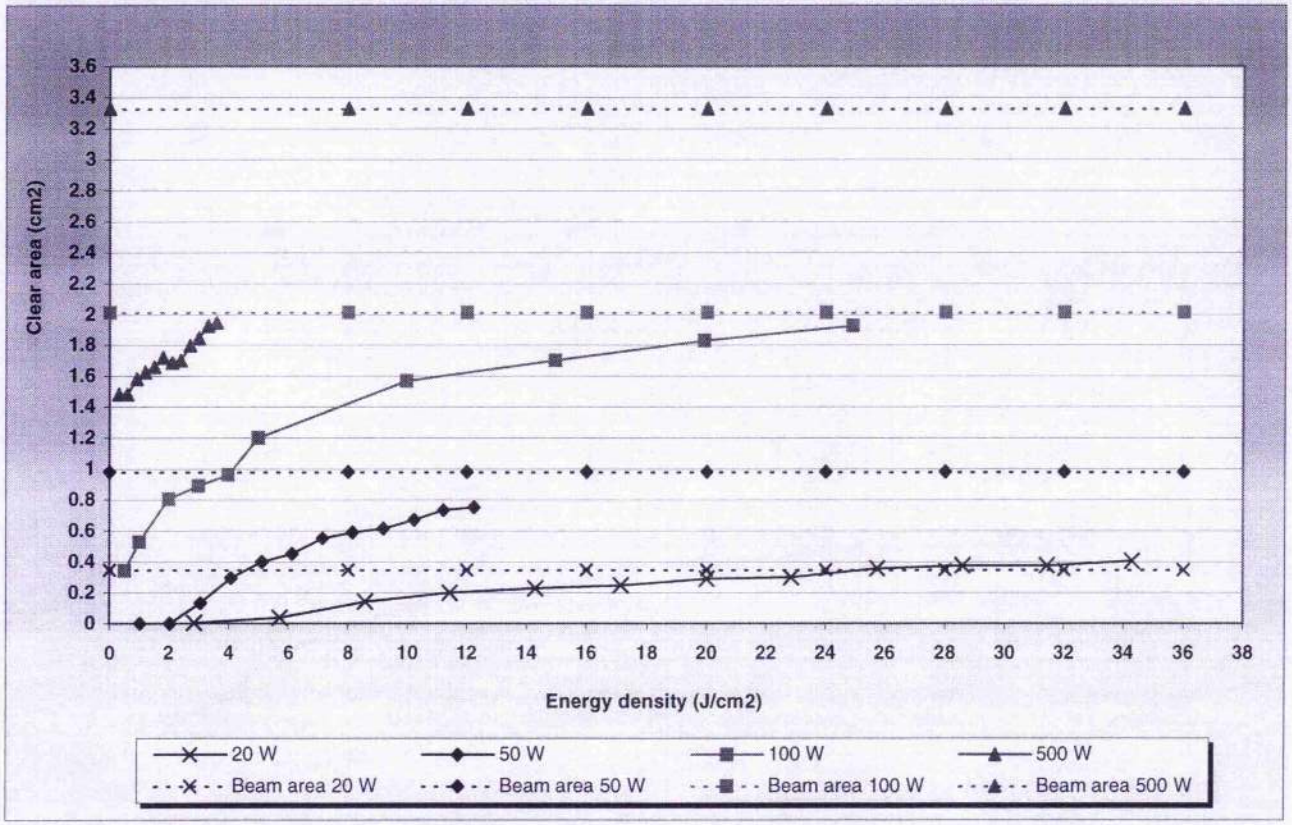


Figure 3-50. Comparison of killing effect of CO_2 laser (continuous wave) at different powers on *M. luteus* on agar plates

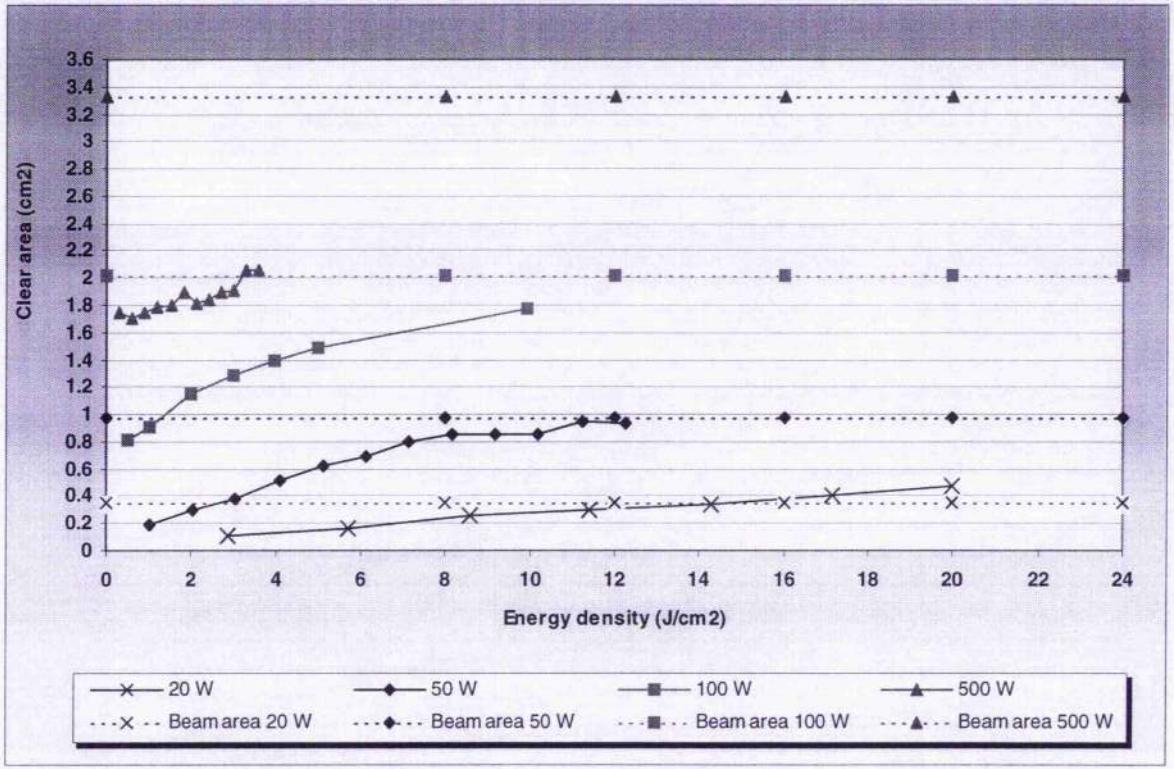


Figure 3-51. Comparison of killing effect of CO_2 laser (continuous wave) at different powers on *S. putrefaciens* on agar plates

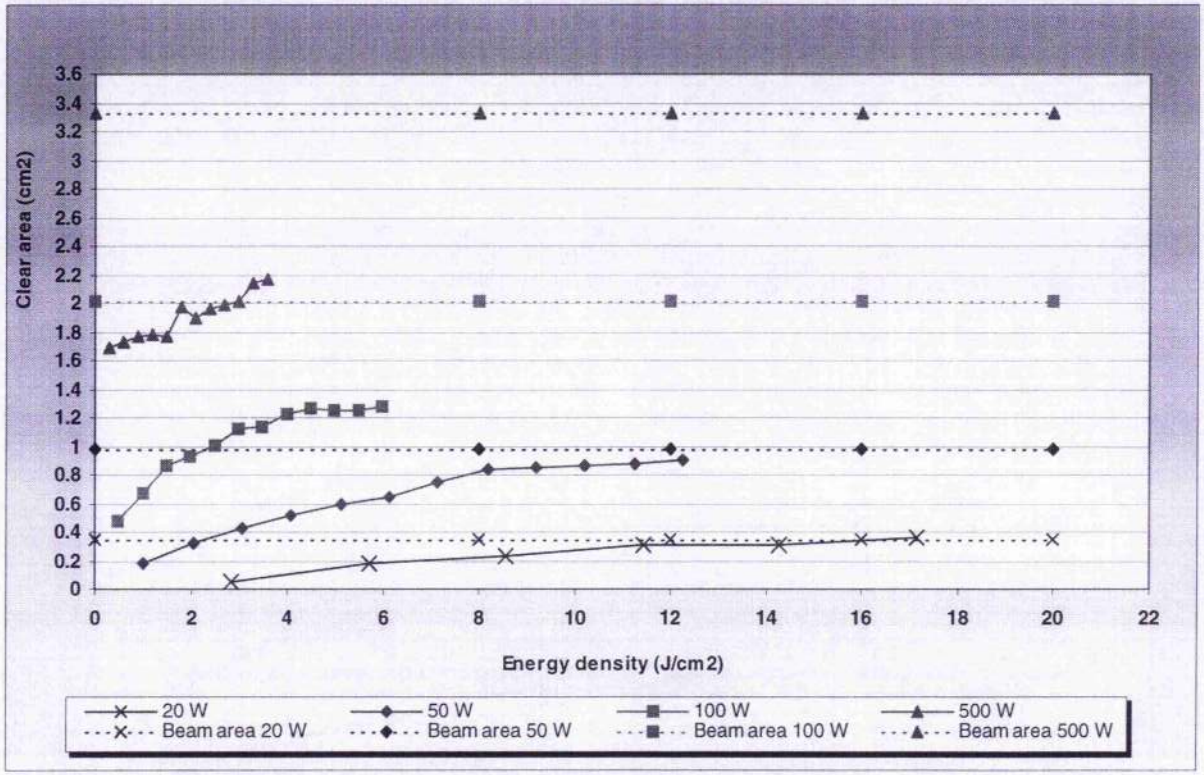


Figure 3-52. Comparison of killing effect of CO_2 laser (continuous wave) at different powers on *L. monocytogenes* on agar plates

3.8 Comparison between viable count and monitored bioluminescence output of E. coli (lux) after different treatments

As *E. coli (lux)* is a bioluminescent strain, it is potentially possible to measure the viability of the cells by measuring the light output instead of by colony counting. This saves valuable time in the experimental process. So, an investigation was done to determine whether there was any correlation between the light output from the *E. coli (lux)* and viable counts after different optical and physical treatments. Suspensions of the bacterium were exposed to UV radiation for 3, 5, 8, 12 and 20 sec at 70 cm distance from the lamps, to Nd:YAG laser radiation for 7, 8, 9, 10 and 11 sec, to microwave radiation for 10 and 15 sec and to conventional heating for 5, 10, 15 and 20 min at 45, 50 and 55°C as described before. Also, the light output from the bacterium after treatment with conventional heating was measured. For combination of treatments, 3 s UV, 9 s laser and 10 s microwave were used and then the light output was measured. A standard curve for light output against colony count was also made by diluting a suspension of *E. coli (lux),* as described in **section 2.3.11.** Results are shown in **Table 3-47** that for this particular *E. coli (lux)* strain grown under standard conditions, the luminometry method can detect down to 1x 10^5 cfu/ml i.e. potentially could measure 4-log reduction in viability.

Table 3-47. Measurement of colony counts and light output for *E. coli (lux)* for the standard curve

No	Cfu/ml	Mean* of light output (Arbitrary units)
1	1×10^9	6735
2	1×10^8	2920
3	1×10^7	328
4	1×10^6	37
5	1×10^5	6.51
6	1×10^4	1.95
7	1×10^3	1.47
8	1×10^2	1.44
9	0	1.40

* No of observation = 3

Table 3-48 shows the results of colony counts and the light output for the bacterium after different treatments. Also, the measured light output and colony counts of the bacterium after different treatments with conventional heating, are shown in **Table 3-49**.

As can be seen in **Table 3-48,** the light output immediately after treatment was not significantly affected by UV treatment even though viability in terms of ability to form colonies on the agar was reduced from about 9 logs to 1 log. In contrast, the laser treatment reduced the light output dramatically, without a corresponding drastic reduction of the subsequent viable counts. The light output decreased from 6600 to 358 units after 8 sec of laser treatment, whereas the colony counts reduced only from 1.3×10^9 to 9.2×10^8 cfu/ml (0.15 log reduction in viability). The relation between the light output and colony counts after treatment with microwave energy was different from that seen with either UV or laser, but was similar to the standard curve. Microwave treatment for 15 sec gave about 3 logs reduction in the viability, whereas the light output reduced from 6600 to about 61. The light output, decreased sharply after the combined treatments and no clear relationship between light output and viable count was obtained. Immediately after treatment of the cells by conventional heating (**Table 3-49**), similar results to laser treatment were observed. Again, the light output reduced dramatically, from about 3000 to 74 units after 5 min treatment at 50°C, whereas no significant reduction in the viability was observed. A more severe treatment, 10 min at 55°C, gave about 3 logs reduction in the viability and the light output reduced to 3 units.

Table 3-48. Correlation between light output and colony count of *E. coli (lux)* after different treatments

No	Treatment	Light output (Arbitrary units)	Colony count (Cfu/ml)
1	Control	6600	1.3×10^9
2	3 sec UV	5800	5.3×10^8
3	5 sec UV	5950	4.6×10^8
4	8 sec UV	7250	1.7×10^8
5	12 sec UV	6350	1.1×10^8
6	20 sec UV	6400	2.5×10^1
7	7 sec laser	3050	1.2×10^9
8	8 sec laser	358	9.2×10^8
9	9 sec laser	12.50	3.2×10^8
10	10 sec laser	2.65	2.6×10^7
11	11 sec laser	1.80	1.5×10^4
12	10 sec microwave	4050	6×10^8
13	15 sec microwave	61.30	3.8×10^6
14	UV + L	26	7.7×10^7
15	L + UV	56.60	4×10^7
16	M + UV + L	2.30	1.6×10^7
17	M + L + UV	2.20	8.1×10^6
18	Saline	1.80	-

Table 3-49. Light output and colony counts of *E. coli (lux)* after different treatments by conventional heating

No	Treatment	Light output (Arbitrary units) 2 h*	Colony count (cfu/ml) 2 h*
1	Control	2860	3.2×10^8
2	5 min at 45^0C	1790	3.2×10^8
3	10 min at 45^0C	920	3×10^8
4	5 min at 50^0C	74	3×10^8
5	10 min at 50^0C	28	2.8×10^8
6	15 min at 50^0C	7.8	2.2×10^8
7	20 min at 50^0C	7.6	1.8×10^8
8	5 min at 55^0C	3.12	1×10^7
9	10 min at 55^0C	3	1.5×10^5

* Measured about 2h after treatment (delivery and preparation time) and then after incubation for 15 min at 37^0C

3.9 Investigation of the killing mechanisms

3.9.1 Effect of released cell constituents on protection of bacteria against UV and laser radiation

In previous experiments, it was seen that after microwave radiation, the viscosity of the solution was increased, presumably due to release of cell constituents. It is possible that such constituents, such as nucleic acids and protein, could absorb UV and laser light and protect surviving bacteria against subsequent exposure to UV and laser radiation.

To investigate this possibility, *S. putrefaciens* and *P. fragi* were chosen for this experiment. As described in section 2.4.1, two supernate fractions were collected from each strain. The first was from a microwave treated suspension of the bacterium for 15 sec and the second was from the untreated suspension. Both supernates were filtered through a 0.2 μm size sterile filter into a sterile universal bottle to insure that they were cell-free. The OD was obtained against normal saline at 260 nm for both solutions. Results are shown in the table below. It is clear that the microwave energy caused release of 260 nm-absorptions material into the supernate and this release was greater with *S. putrefaciens* than *P. fragi*. 1 ml of fresh bacterial suspension, which was made separately, was pipetted into each solution and viable cell counts were made.

Bacterium	OD_{260} for treated supernate	OD_{260} for untreated supernate
S. putrefaciens	**0.647**	**0.060**
P. fragi	**0.217**	**0.077**

Then, 1 ml from the suspensions was taken for subsequent treatments. The treatment parameters used for each bacterium are shown below:

Parameters used for *S. putrefaciens*			Parameters used for *P. fragi*		
UV	3 sec at 60 cm	1620 μW s/cm^2	UV	3 sec at 70 cm	1470 μW s/cm^2
Laser	6 sec	414.6 J/cm^2	Laser	8 sec	552.8 J/cm^2

Results of this experiment are shown in **Table 3-50** for *S. putrefaciens* and **Table 3-51** for *P. fragi*.

Although it should be borne in mind that the results are from a single experiment they showed that released constituents could possibly protect bacteria against subsequent treatments. In all cases, the reduction in viability was less in the samples where the bacteria were suspended in supernate from microwave-treated cells rather than in supernate from untreated cells.

S. putrefaciens: Treatment of the control suspension by 6 sec laser followed by 3 sec UV radiation gave a total log reduction in viability of 1.62, but with the suspension containing the released cell constituents the log reduction in viable counts was about 1.5 **(Table 3-50).** With the other sequence of treatment, 3 sec UV then 6 sec laser, the log reduction was 2.12 for the cells suspend in the untreated supernate, whereas the value was 1.45 logs for the cells suspended in the treated supernate. Thus, the microwave-treated supernate with an OD of about 0.65 appeared to protect cells against subsequent sequential treatments between 0.1 – 0.67 logs than the untreated supernate with OD 0.06.

P. fragi: Similar to the previous bacterium, this organism released constituents after treatment of the bacterial suspension with the microwave radiation but to a lesser extent, and the result by supernate was again able to protect fresh cells against the subsequent treatments. Treatment of the control suspension by 8 sec laser followed by 3 sec UV radiation gave a total log reduction in viability of 1.83, but with the suspension containing the released cell constituents the log reduction in viable counts was about 1.4. With the other sequence of treatments, 3 sec UV then 8 sec laser, the log reduction was about 1.2 for both the cells suspended in the untreated supernate and treated supernate. The differences between the log reduction by subsequent treatments on cells suspended in the treated and untreated supernate were between 0.01–0.49 logs (**Table 3-51**). These values were less than the values for *S. putrefaciens*, which may be, however, due to differences between the bacteria or to the higher OD of the microwave-treated supernate sample from *S. putrefaciens*.

Table 3-50. Effect of microwave-released cell constituents on protection of *S. putrefaciens* against subsequent treatments

Control	First treatment				Second treatment				
	Treatment	Mean of survivor bacteria (cfu/ml)	Mean of log reduction cfu/ml	STDEV*	Treatment	Mean of survivor bacteria (cfu/ml)	Mean of log reduction cfu/ml	Mean of total log reduction cfu/ml	STDEV
Treated supernate containing 2×10^8 cfu/ml	6 sec laser	1.6×10^7	0.78	0.11	3 sec UV	1.2×10^6	0.73	1.51	0.10
	3 sec UV	2.2×10^7	0.64	0.01	6 sec laser	2.3×10^6	0.81	1.45	0.44
Untreated supernate containing 1.8×10^8 cfu/ml	6sec laser	1.1×10^7	0.84	0.04	3 sec UV	6.8×10^5	0.78	1.62	0.03
	3 sec UV	1.4×10^7	0.81	0.09	6 sec laser	1.6×10^5	1.31	2.12	0.16

*STDEV: Standard deviation (n=3)

Table 3-51. Effect of microwave-released cell constituents on protection of *P. fragi* against subsequent treatments

Control	First treatment				Second treatment				
	Treatment	Mean of survivor bacteria (cfu/ml)	Mean of log reduction cfu/ml	STDEV*	Treatment	Mean of survivor bacteria (cfu/ml)	Mean of log reduction cfu/ml	Mean of total log reduction cfu/ml	STDEV
Treated supernate containing 1.7×10^8 cfu/ml	8 sec laser	2.2×10^7	0.59	0.01	3 sec UV	3.2×10^6	0.75	1.34	0.26
	3 sec UV	2.1×10^7	0.61	0.04	8 sec laser	6.2×10^6	0.58	1.19	0.31
Untreated supernate containing 2.5×10^8 cfu/ml	8 sec laser	1.3×10^7	0.94	0.13	3 sec UV	5.7×10^5	0.88	1.83	0.1
	3 sec UV	1.4×10^7	0.82	0.01	8 sec laser	4.7×10^6	0.39	1.21	0.09

*STDEV: Standard deviation (n=3)

3.9.2 Effect of different treatments on release of nucleic acids and protein from bacterial suspensions

It is likely that some of the killing treatments used in these experiments will rupture the cell envelope and release constituents such as DNA, RNA and protein. Some treatments may be more effective in causing release than other treatments and measurement of the released material may show differences between the killing mechanisms by the different treatments. To investigate this, it was decided to expose the *E. coli (lux)* suspension to different treatments and then measure the OD of the supernates at 260 nm (for DNA and RNA) and 280 nm (for protein). Reductions in viable count by the different methods were also measured. Thus, bacterial suspensions were exposed to the following individual treatments:

Microwave: 12, 14, 16 and 18 sec
Conventional heating: 5 min at 45, 50, 55 and 60^{0}C
UV: 8, 10, 12 and 14 sec at 80 cm
Laser: 8, 9, 10 and 11 sec
Ozone: 5, 10, 15 and 20 min
Combination: 10 sec UV, 9 sec laser and 5 min conventional heating at 55^{0}C

Optical density readings of the resulting cell supernates were measured as described in Section 2.4.3 for all suspensions. The means of the results are shown in **Figures 3-53 and 3-54** for different individually treatments **and in Figures 3-55 and 3-56** for combined treatments. The OD of the supernate from the control suspension with no treatment was 0.075 at 260 nm and 0.052 at 280 nm. Results showed that, with all treatments, the greater the killing effect produced the greater the amount of released material. As can be seen in **Figure 3-53**, killing by conventional heating gave greater nucleic acid release than the other treatments. After a 2-log reduction in viable count, the OD was about 0.25 for conventional heating, 0.21 for laser, 0.17 for microwave and ozone and 0.09 for UV radiation. After a 4.5 log reduction in viable count, the values were increased to 0.31 for conventional heating, 0.21 for laser and microwave radiation, 0.19 for ozone.

Similar results but with lower levels of release were obtained when the OD at 280 nm was monitored, which indicates the level of released protein in the suspension. After a 2-log reduction in viable counts by conventional heating, the OD reading was 0.121, whereas the value for the laser treatment was 0.115 and almost 0.09 for ozonation and microwave radiation. After a 2 log reduction in viable count by UV radiation the OD was 0.054, which was similar to the control suspension. After a 4-log reduction in viability, the OD was 0.141 for conventional heating, 0.12 for laser, 0.92 for ozone and microwave radiation and about 0.07 for UV radiation. Killing by more than 5-log reduction in viable counts was studied only for conventional heating, laser and microwave radiation. After about a 6-log reduction in viable counts, the OD was 0.148 for conventional heating, 0.138 for laser radiation and 0.135 for microwave radiation. From both figures, it is clear that for a given reduction in viability, conventional heating followed by laser caused most release of cell constituents. Microwave and ozonation gave similar levels of release whereas UV caused little release.

As can be seen in **Figures 3-55 and 3-56** with combination of two or three treatments there was little difference observed in the OD at either 260 or 280 nm for the different treatments. With the combination of 3 treatments, although the difference in OD between the different sequences was small, the final OD value for the order, H + UV + L was slightly greater than for the order L + H + UV at both 260 and 280 nm.

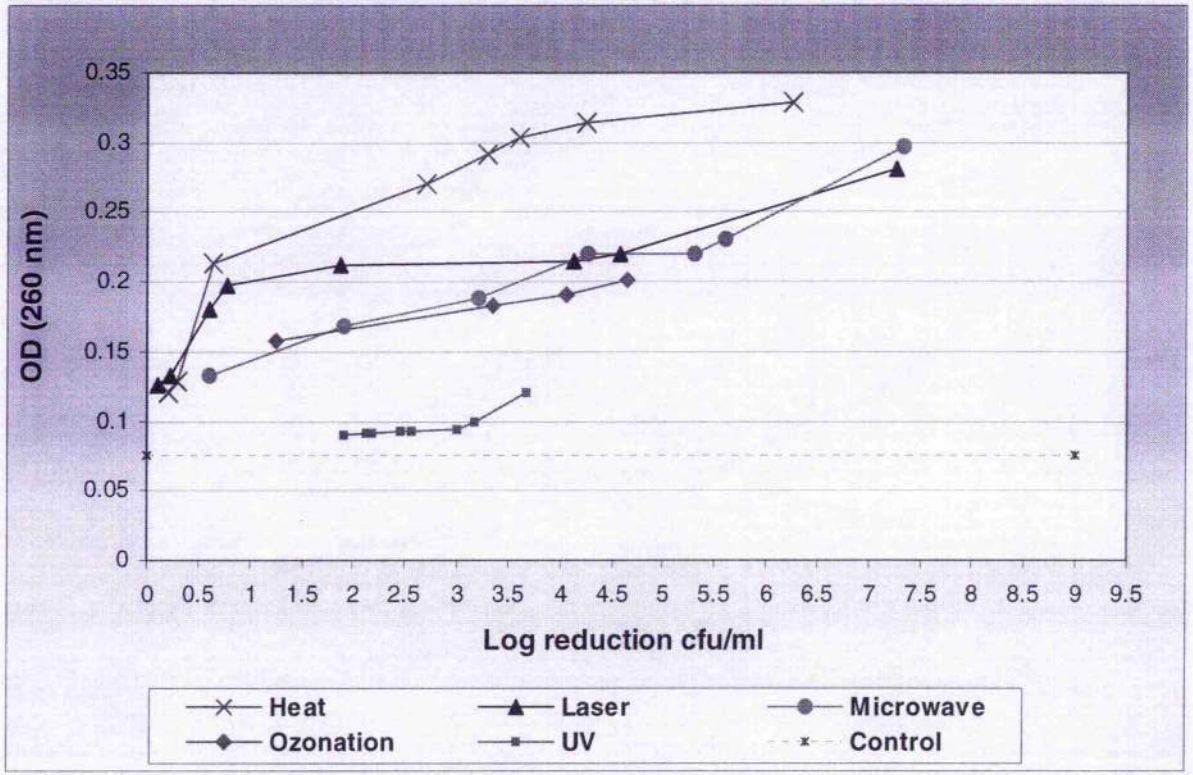


Figure 3-53. Effect of different treatments on killing and release of cell constituents from *E. coli (lux)* in suspension (1)

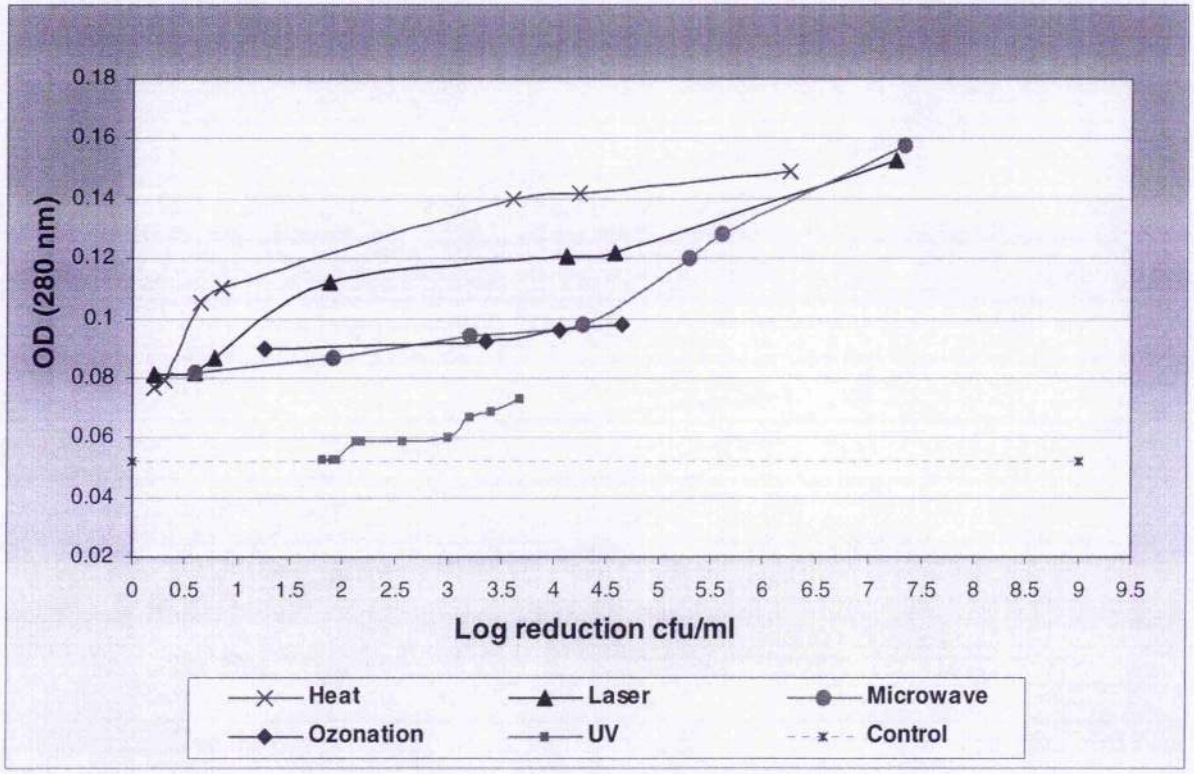


Figure 3-54. Effect of different treatments on killing and release of cell constituents from *E. coli (lux)* in suspension (2)

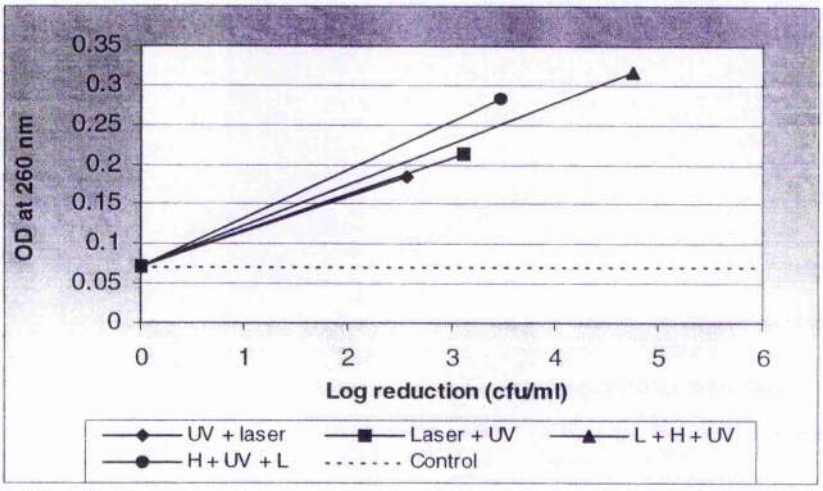


Figure 3-55. Effect of combined treatments on killing and release of cell constituents from *E. coli (lux)* in suspension (OD_{260})

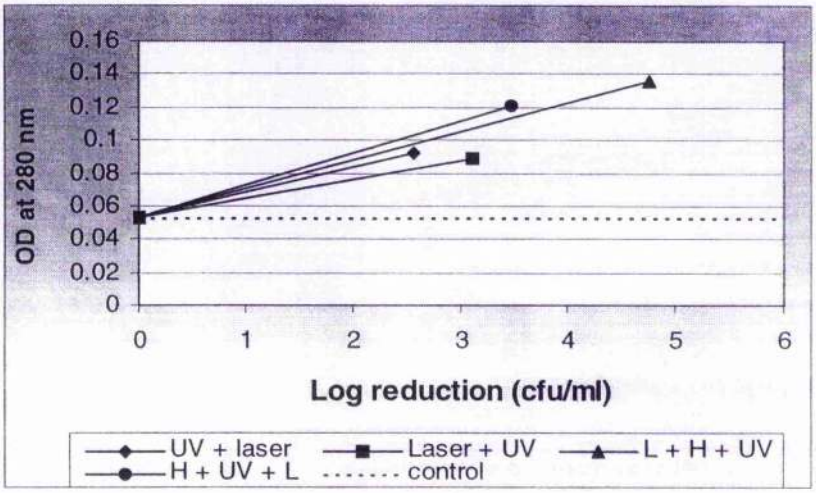


Figure 3-56. Effect of combined treatments on killing and release of cell constituents from *E. coli (lux)* in suspension (OD_{280})

3.9.3 Investigation of the effect of different cooling methods after microwave treatment on the effectiveness of subsequent treatments

This experiment was designed to find out if a cold-shock, applied by different methods, could increase the killing effect of subsequent treatments. Suspensions in saline were initially exposed to a mild microwave treatment or left untreated. A Gram-positive bacterium (*M. luteus*) and a Gram-negative bacterium (*E. coli, lux*) were chosen. Cooling at room temperature, by ice and by a quick freezing method in a mixture of acetone and dry ice were investigated. After cooling, the suspensions were allowed to warm to room temperature. As described in section 2.4.2, the bacteria were then exposed to a single treatment (Nd:YAG laser) after cooling. Based on sensitivity of the bacteria, *M. luteus* was exposed to laser for 10 sec and *E. coli (lux)* for 9 sec. Results of the experiment are shown in **Tables 3-52 and 3-53**. Cold shock after microwave treatment gave some further reduction in viable counts of *E. coli* compared with microwave treated bacteria allowed to cool to room temp. The value for cooling by ice was 0.26 log, whereas for freezing by acetone and dry ice was 0.56 log. These values can be compared with 0.17 and 0.35 log reduction respectively, when the suspension had not been treated by microwave (**Table 3-52**, first grey column). No significant reduction in viable count of *M. luteus* was apparent when the bacterial suspension, with no pre-treatment by microwave, was exposed to cold shock with the ice or mixture of acetone and dry ice. When the suspension had been pre-treated by microwave, the cooling by ice or freezing mixture caused about 0.13 log reduction in the viability (**Table 3-53**, first grey column).

After treatment by microwave and a cold shock by mixture of dry ice and acetone, killing by laser increased to 1.52 and 1.03 log reduction in viability for *E. coli* and *M. luteus* respectively whereas, without microwave treatment, killing was 1.25 for *E. coli* and 0.97 for *M. luteus* (second grey column in **Tables 3-52 and 3-53**). The killing effect of laser, after cooling by ice and cooling by room temperature was almost the same for both bacteria. The values were between 0.73 to 0.95 log reduction in viable counts when the suspension was treated with microwave and 0.52 to 0.59 logs without microwave treatment (second grey column in **Tables 3-52 and 3-53**).

The total log reduction in viability, by cooling and laser, for both bacteria was always greater when the bacterial suspension had a cold shock by mixture of dry ice and acetone

in comparison to other cooling methods. The log reduction was always greater for *E. coli* than for *M. luteus* (last column in **Tables 3-52 and 3-53**).

It was concluded that probably a rapid cooling of the bacterial suspension after microwave treatment or perhaps after other heat methods not only could have enhanced the killing effect on the bacteria but also may have increased their susceptibility to the killing effect of subsequent treatment(s).

Table 3-52. Effect of different cooling methods after microwave treatment on the viability of *E. coli (lux)* in suspension

	Microwave Surviving bacteria Cfu/ml	Log reduction cfu/ml	STDEV	*Type of cooling* Surviving bacteria (cfu/ml)	Log reduction cfu/ml	STDEV	*Treatment* Surviving bacteria (cfu/ml)	Log reduction cfu/ml	STDEV	Total log reduction by cooling and laser (cfu/ml)
Control: 4.9×10^8 cfu/ml	15 sec microwave 3.2×10^8	0.18	0.33	*Room temp.* 3.2×10^8	0	0.15	*9 sec laser* 3.5×10^7	0.95	0.20	**0.95**
				Ice 1.7×10^8	0.26	0.28	*9 sec laser* 3.1×10^7	0.75	0.26	**1.01**
				Dry ice + acetone 8.8×10^7	0.56	0.09	*9 sec laser* 2.6×10^6	1.52	0.52	**2.08**
Control: 4.5×10^8 cfu/ml	No microwave	-	-	*Room temp.* 3.1×10^8	0.15	0.21	*9 sec laser* 8.1×10^7	0.59	0.41	**0.74**
				Ice 3×10^8	0.17	0.08	*9 sec laser* 8×10^7	0.58	0.42	**0.75**
				Dry ice + acetone 2×10^8	0.35	0.18	*9 sec laser* 1.1×10^7	1.25	0.12	**1.60**

Table 3-53. Effect of different cooling methods after microwave treatment on the viability of *M. luteus* in suspension

	Microwave Surviving bacteria (cfu/ml)	Log reduction cfu/ml	STDEV	*Type of cooling* Surviving bacteria (cfu/ml)	Log reduction cfu/ml	STDEV	*Treatment* Surviving bacteria Cfu/ml	Log reduction cfu/ml	STDEV	Total log reduction by cooling and laser (cfu/ml)
Control: 9×10^7 cfu/ml	15 sec microwave 5.2×10^7	0.23	0.02	*Room temp.* 5.2×10^7	0	0.02	*10 sec laser* 7×10^6	0.87	0.13	**0.87**
				Ice 4×10^7	0.12	0.11	*10 sec laser* 7.5×10^6	0.73	0.03	**0.85**
				Dry ice + acetone 3.9×10^7	0.14	0.12	*10 sec laser* 3.6×10^6	1.03	0.72	**1.17**
Control: 6.1×10^7 cfu/ml	No microwave	-	-	*Room temp.* 6.7×10^7	- 0.04	0.13	*10 sec laser* 1.8×10^7	0.58	0.58	**0.54**
				Ice 5.6×10^7	0.03	0.15	*10 sec laser* 1.7×10^7	0.52	0.39	**0.55**
				Dry ice + acetone 5.2×10^7	0.07	0.11	*10 sec laser* 5.4×10^6	0.97	0.86	**1.04**

3.9.4 Effect of different treatments on the sensitivity of *E. coli(lux)* to lysis by SDS

In a previous experiment it was observed that different treatments on *E. coli* in suspension had different effects on the release of cell contents. An alternative way to investigate cell envelope damage, caused by different treatments is to measure the sensitivity of the treated cell to subsequent lysis by a 0.1% solution of SDS (**219**). In this method, the bacterial suspension was treated and the OD of the suspension at 600 nm was measured before and at different times after exposure to SDS. The hypothesis is that more lysis will occur with more injured cells and so, a greater reduction in OD will be observed. To compare the results, a standard curve was obtained by exposure of a fresh bacterial suspension (*E. coli*) to 0.1% SDS (**Figure 3-57**) and no other treatment. As can be seen in the figure, after incubation of the suspensions for 30 min at 37°C, the OD for the suspension with no SDS showed some increase. The OD then slightly reduced with time. For the suspension containing SDS, a small reduction in the OD was observed with time.

Suspensions of *E. coli (lux)* were exposed to different treatments and energies as shown below:

Microwave: 12, 15 and 18 sec
Conventional heating: 5 min at 45, 55 and 65°C
UV: 8, 12 and 16 sec at 80 cm
Laser: 8, 10 and 12 sec
Ozone: 2, 5, 10 and 15 min

Results for the different treatments are shown in **Figures 3-58 to 3-62**. Perhaps due to multiplying cells, the OD showed a small initial increase when the cells were incubated for 30 min without SDS. This occurred in the control (untreated) sample (**Figure 3-57**) and with all treatments except ozonation. After the initial increase or decrease, the OD was fairly stable for all treatments with increasing time. With suspensions treated with SDS the pattern was different. When SDS was added to the suspension treated for 5 min

by conventional heating at 45°C, the OD slightly decreased (from 0.09 to 0.08) up to 3h. A large reduction in OD was observed after exposure to SDS for 30 min, in suspensions, which had been treated at 55 and 65°C by conventional heating, followed by SDS exposure. The initial OD was 0.09 and, after 30 min of exposure to SDS, had reduced to 0.045 and 0.02 respectively. The values then reduced to about 0.03 and 0.01 at 3h **(Figures 3-58).**

Similar patterns were obtained for suspension, which had been exposed to the more severe microwave and laser treatments (**Figures 3-59 and 3-60**).

With UV and ozone treatments, the observations were different. No increase in OD (from 0.14) was observed when the cells were treated with ozone and incubated for 30 min without SDS, whereas, after that, a slight reduction in OD was apparent. With the suspensions which had been treated by ozone, followed by SDS exposure, there was a decrease from about 0.15 to 0.08 in OD after 3h incubation, but this reduction was about half of that observed for treatment by conventional heating and laser.

No significant differences in OD were seen for suspensions previously treated with UV then incubated with or without SDS.

Based on the above results, it was concluded that cells treated by laser, microwave and conventional heating are more sensitive to lysis by 0.1% SDS than cells treated with ozone and UV. The results clearly showed that laser, microwave or conventional heating have some effect on the cell envelope and sensitise the bacteria to lysis by SDS. These effects, however, are probably lower for ozone and may be minimal for UV, which is well known to cause damage primarily to the DNA of the cell.

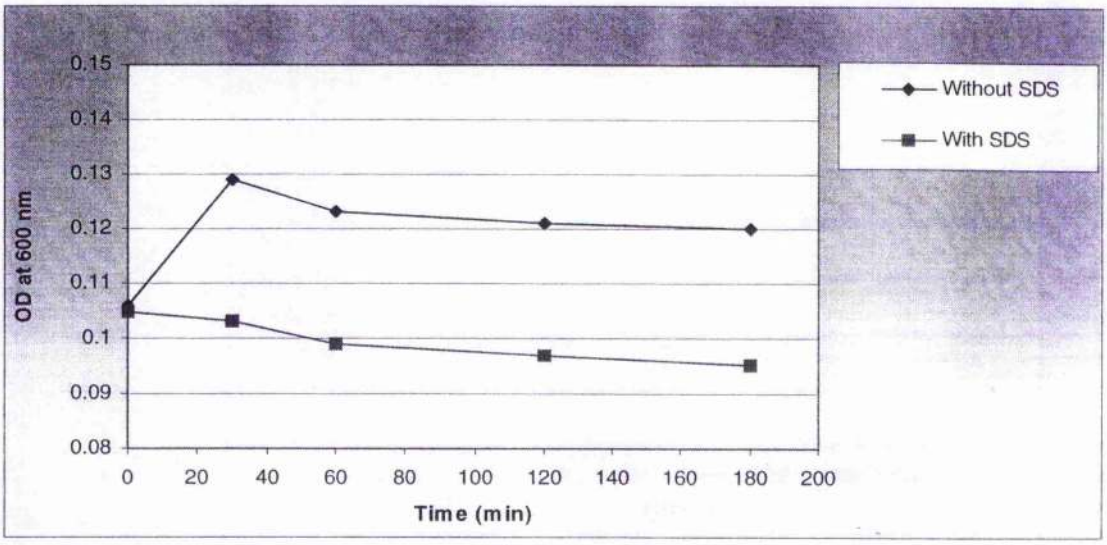


Figure 3-57. Lysis of an untreated (control) suspension of *E. coli (lux)* by SDS

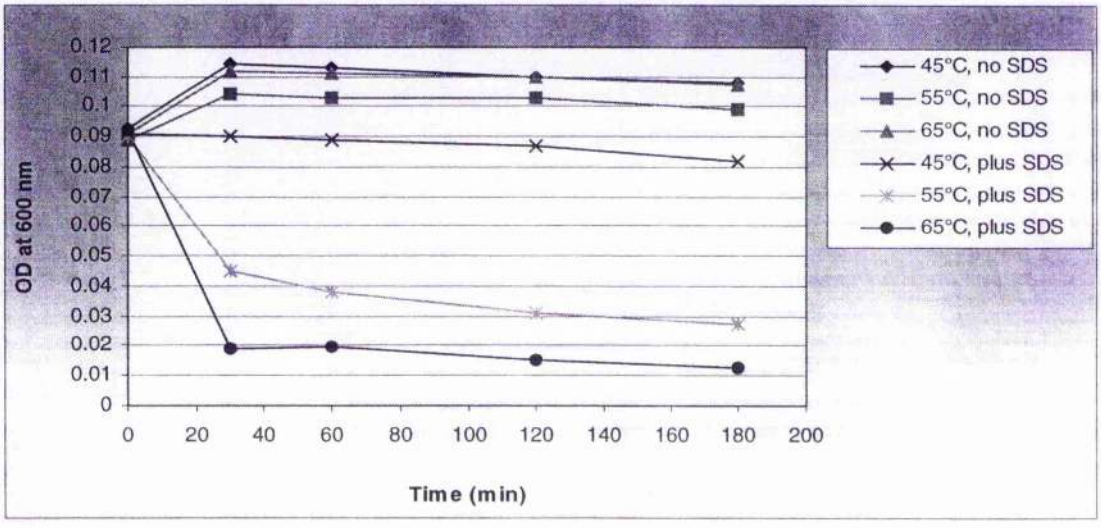


Figure 3-58. Lysis of *E. coli (lux)* by SDS after treatment by conventional heating

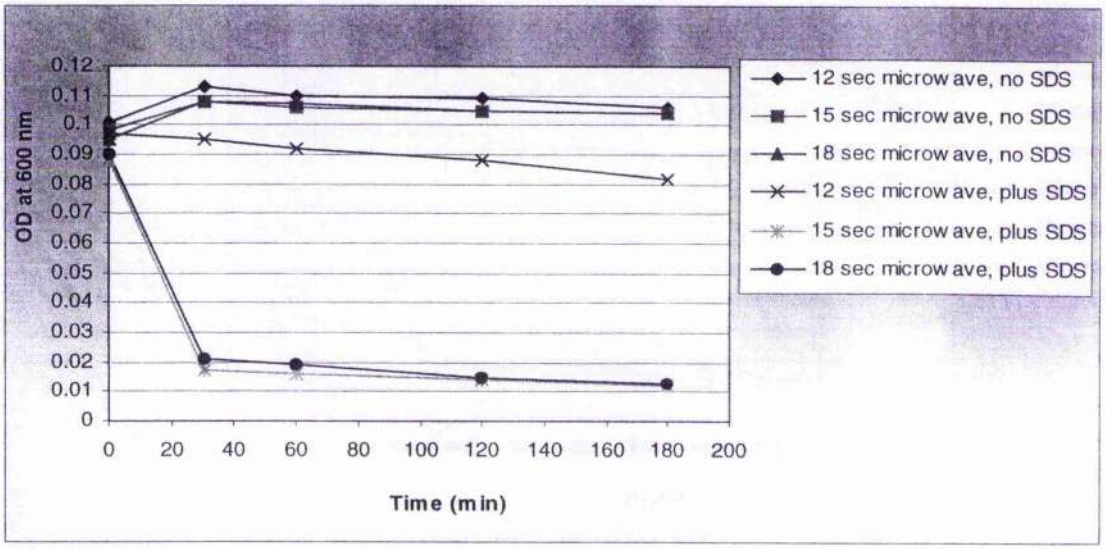


Figure 3-59. Lysis of *E. coli (lux)* by SDS after treatment by microwave radiation

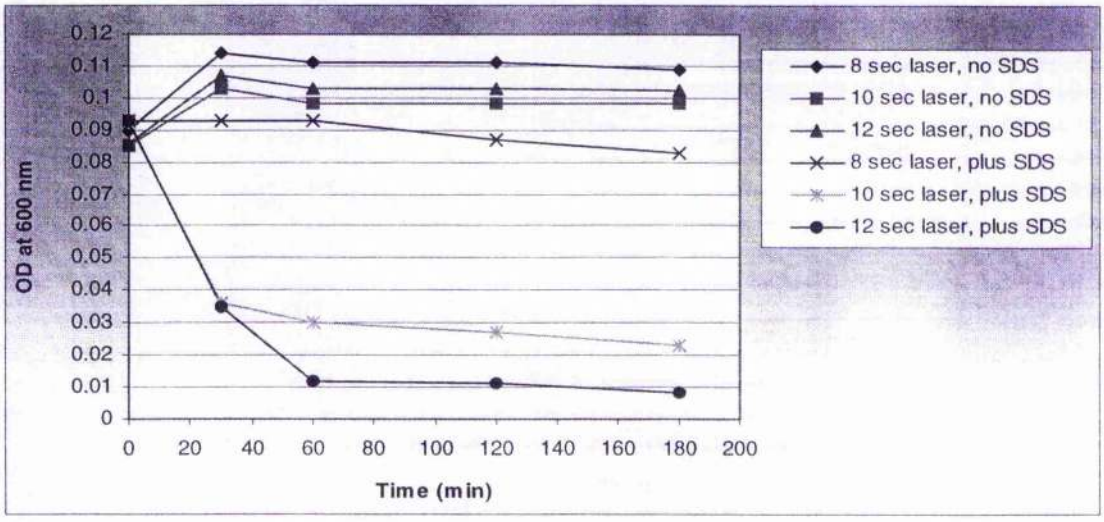


Figure 3-60. Lysis of *E. coli (lux)* by SDS after treatment by laser light

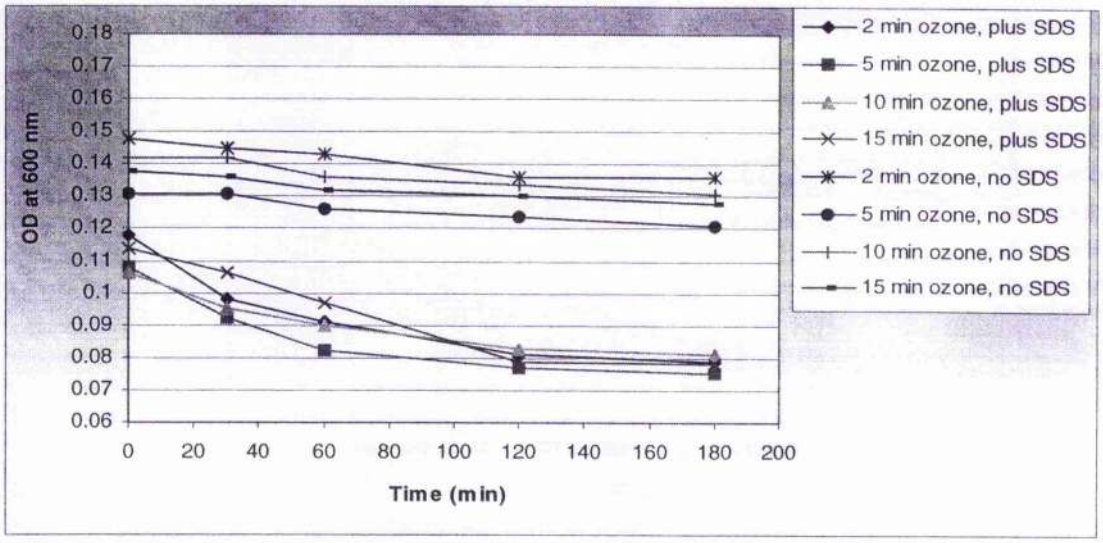


Figure 3-61. Lysis of *E. coli (lux)* by SDS after treatment by %0.1 solution of ozone

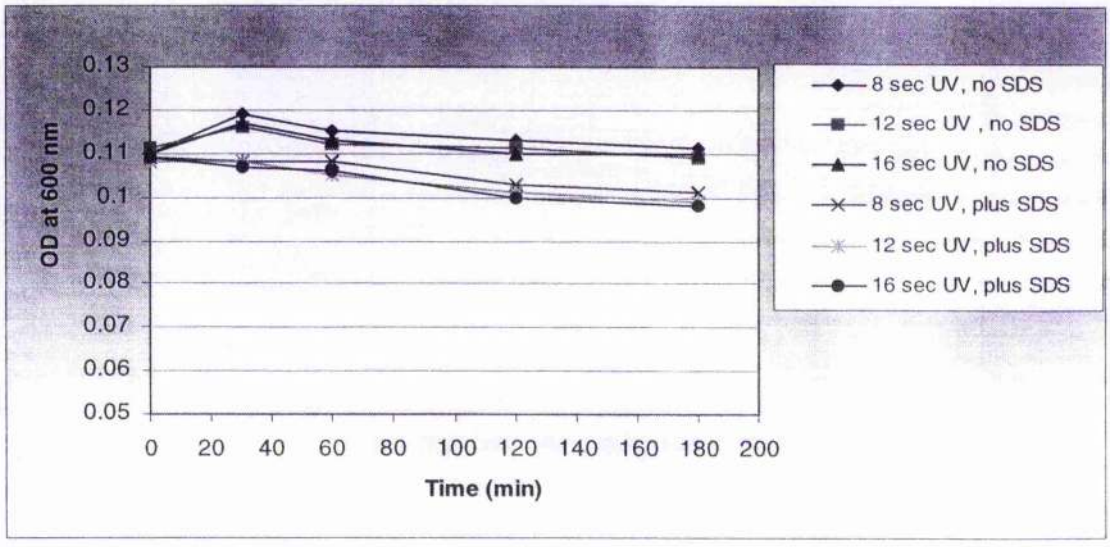


Figure 3-62. Lysis of *E. coli (lux)* by SDS after treatment by UV radiation

3.9.4.1 Killing effect of 0.1% SDS on stressed cells and correlation with reduction of OD after incubation

In the previous experiment it was found that when bacterial suspensions, treated by different methods, were exposed to SDS 0.1%, the OD clearly showed a reduction. Untreated cells showed a little reduction in OD with SDS alone. It was decided to investigate whether pre-treatment, such as by mild heating, ozone, laser, etc, then exposure to SDS had a synergistic effect on bacterial killing. Bacterial suspensions (*E. coli*) after different treatments were incubated in SDS 0.1% and also in normal saline for up to 60 min. Colony counts and OD measurement were made before and after exposure to SDS for 30 and 60 min. As in the previous experiment, *E. coli (lux)* was exposed to following treatments:

Nd:YAG laser: for 10 sec
Ozone: for 2 min
UV: 3x30 W lamp, at 80 cm for 10sec
Conventional heating: 55°C for 5 min

The treated cells were then incubated in SDS 0.1% or normal saline for 60 min as described. Results are shown in **Table 3-54**. **Figure 3-63** shows the log reductions in viable counts and OD_{600} after treatment by various methods, and then incubation in SDS and normal saline for 60 min. As can be seen, the reductions in the viability and OD at 600 nm in suspensions, subsequently incubated with normal saline were very small. Control suspension incubated with SDS also gave a small reduction in viability, compared to the control suspension incubated with normal saline, which showed bacterial growth after 60 min. When pre-treated bacteria were exposed to SDS 0.1% however, the subsequent reduction in OD at 600 nm and viability were consistently greater. For example, the effects were most marked with ozone treatment. In cells treated with ozone, the OD_{600} reduced from 0.162 to 0.093 in suspension containing SDS, whereas in suspension containing saline the OD_{600} was reduced from 0.161 to 0.158. The killing effect also was greater than the sum of the effects with either treatment alone. It may be

that SDS had a greater killing effect on stressed cells. Thus, the killing effect of different concentration of SDS was investigated. Also, it was decided to repeat the experiment with *L. monocytogenes*, to find out whether the killing effect by SDS of stressed bacteria was also applicable to a Gram-positive species.

Table 3-54. Comparison of killing effect and change in OD_{600} of *E. coli (lux)* by SDS 0.1% in combination with other treatments

N0.	Treatments	OD_{600} before incubation	Surviving bacteria before incubation (cfu/ml)	OD_{600} after incubation in (±SDS) for 30 min	Surviving bacteria after incubation for 30 min in (±SDS) (cfu/ml) Log reduction in viability	OD_{600} after incubation in (±SDS) for 60 min	Surviving bacteria after incubation for 60 min in (±SDS) (cfu/ml) Log reduction in viability
1	Control, plus saline	0.167	7.5×10^7	0.170	8×10^7 -0.03	0.166	8.1×10^7 -0.03
2	Control, plus SDS	0.160	7.5×10^7	0.158	7×10^7 0.03	0.151	7×10^7 0.03
3	10 sec laser, plus saline	0.159	2.5×10^6	0.159	2.4×10^6 0.02	0.152	1.5×10^7 0.22
4	10 sec laser, plus SDS	0.163	2.5×10^6	0.148	1.5×10^6 0.22	0.139	1×10^6 0.04
5	2 min ozonation, plus saline	0.161	1×10^5	0.160	8×10^4 0.09	0.158	5×10^4 0.30
6	2 min ozonation, plus SDS	0.162	1×10^5	0.136	4.5×10^4 0.34	0.093	3×10^4 0.52
7	10 sec UV, plus saline	0.157	1.1×10^4	0.153	1×10^4 0.04	0.152	1×10^4 0.04
8	10 sec UV, plus SDS	0.160	1.2×10^4	0.154	1×10^4 0.08	0.143	5×10^3 0.38
9	5 min heat, plus saline	0.163	6×10^6	0.160	5.9×10^6 0.01	0.159	5.9×10^6 0.01
10	5 min heat, plus SDS	0.168	6×10^6	0.142	5.5×10^6 0.04	0.123	5.1×10^6 0.07

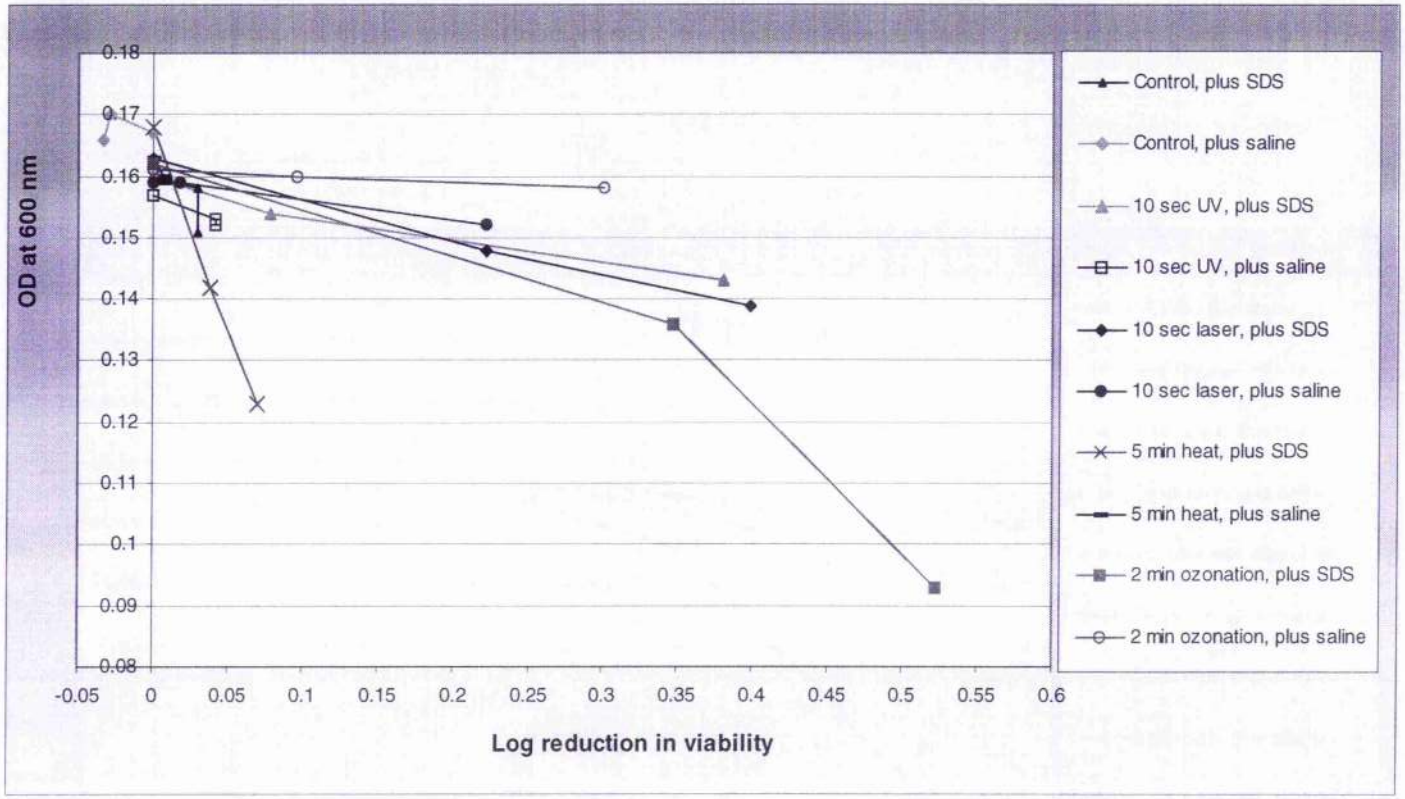


Figure 3-63. Effect of SDS in combination with other treatments on OD_{600} and viability of *E. coli (lux)* at 60 min

3.9.4.2 Killing effect of SDS on *Listeria monocytogenes*

As in the previous experiment, this experiment was designed to investigate any synergistic effects of SDS on killing of bacterial cells by other methods. *Listeria monocytogenes* was exposed to different treatments and the reductions in viability and OD_{600} were determined after exposure to SDS 0.1% or to normal saline for 60 min. First, the cells were treated with different treatments as described.

Nd:YAG laser: for 10 sec
Ozone: for 2 min
UV: 3x30W lamp, at 80 cm for 12 sec
Conventional heating: 55°C for 5 min

The treated cells were then incubated in the SDS 0.1% or normal saline for 60 min. Colony counts were made after 30 min and also the OD was obtained at 600 nm. With *L. monocytogenes*, a large reduction (more than 4.9 log reduction) in viability was found when untreated cells were exposed to SDS 0.1% for 60 min; the OD_{600} was reduced from 0.101 to 0.085 after 60 min. No significant reduction in viability (0.18 log) was apparent in untreated cells incubated in normal saline for 60 min. It was concluded that, in contrast to *E. coli (lux), L. monocytogenes* was very sensitive to SDS 0.1%. The above experiment suggested investigating the killing effect of different concentrations of SDS on *E. coli (lux)* and *L. monocytogenes*.

As *L. monocytogenes* was killed rapidly by SDS 0.1%, the bacterium was exposed to various low SDS concentrations and then combination of SDS with other treatments was investigated.

The cells were incubated in 0.01, 0.005 and 0.1% SDS and colony counts were made after 15 and 30 min. Results are shown in **Table 3-55**. Again, the results showed that the bacterium was highly sensitive to SDS. After incubation in SDS 0.01% for 30 min, more than 4 log reduction in viable count was achieved, whereas 15 min incubation gave about

a 0.2 log reduction in the viability. In higher concentrations, no viable bacteria were detected.

To find out whether there was any synergistic effect of different treatments such as heat, laser, *etc.* and SDS on *L. monocytogenes*, the bacterium was exposed to different treatments and then incubated in SDS 0.01% for 15 min. Colony counts were made before and after incubation and the OD_{600} values were obtained as described previously.

Results are shown in **Table 3-56**. As can be seen, the treated cells incubated in SDS were killed more than the cells incubated in normal saline. Control suspensions, incubated in SDS 0.01% and saline for 15 min, showed a 0.4 and 0.09 log reduction respectively in viable count. The best result was achieved by combination of laser and SDS. More than a 2 log reduction in viability was found when laser-treated cells were exposed to SDS 0.01% for 15 min, whereas in laser-treated cells incubated in normal saline only 0.04-log reduction in viability was achieved. In combinations of UV or conventional heating with SDS, a greater reduction in viability was also apparent. A 1.67 and 1.46 log reduction in viability was achieved for the cells pre-treated with conventional heating and UV respectively, then incubated in SDS 0.01% for 15 min. The reduction for either the UV or heated pre-treated cells, incubated in normal saline was 0.17-log. Only a small reduction in viability (0.45 log), in comparison to the control suspension, was achieved for the ozone pre-treated cells incubated in SDS. The value for the cells incubated in normal saline was 0.07 log reduction in viability. Results clearly showed that, although low concentrations of SDS were effective in killing *L. monocytogenes,* the combination with other methods such as laser, UV and conventional heating improved the killing effect. This suggests that combination of a low concentration SDS with laser, UV or heating could be useful for decontamination of bacteria on materials and surfaces.

Table 3-55. Effect of different concentration of SDS on *L. monocytogenes*

SDS concentration (%)	Surviving bacteria before incubation (cfu/ml)	Surviving bacteria after incubation for 15 min (cfu/ml)	Log reduction in cfu/ml	Surviving bacteria after incubation for 30 min (cfu/ml)	Log reduction in cfu/ml
0.01	**2×10^8**	**1.2×10^8**	**0.22**	**1.1×10^4**	**4.26**
0.05	**2×10^8**	**<2500**	**>4.90**	**<2500**	**>4.90**
0.1	**2×10^8**	**<100**	**>6.30**	**<100**	**>6.30**

Table 3-56. Effect of various treatments and SDS on *L. monocytogenes*

No	Treatments	OD before incubation in SDS	Surviving bacteria before incubation (cfu/ml)	OD after incubation in SDS for 15 min	Surviving bacteria after incubation for 15 min (cfu/ml)	Log reduction in cfu/ml
1	Control, plus SDS	0.366	2.5×10^8	0.368	1.0×10^8	0.40
2	Control, plus saline	0.362	2.5×10^8	0.360	2.0×10^8	0.09
3	10 sec laser, plus SDS	0.308	2.0×10^8	0.316	1.5×10^6	2.12
4	10 sec laser, plus saline	0.307	2.0×10^8	0.309	1.8×10^8	0.04
5	2 min ozonation, plus SDS	0.314	6.0×10^6	0.317	2.1×10^6	0.45
6	2 min ozonation, plus saline	0.314	6.0×10^6	0.316	5.1×10^6	0.07
7	12 sec UV, plus SDS	0.329	1.5×10^8	0.332	5.2×10^6	1.46
8	12 sec UV, plus saline	0.320	1.5×10^8	0.328	1.0×10^8	0.17
9	5 min heat, plus SDS	0.322	1.4×10^8	0.328	3.0×10^6	1.67
10	5 min heat, plus saline	0.313	1.4×10^8	0.310	9.5×10^7	0.17

Parameters used:

Nd:YAG laser: for 10 sec
Ozone: for 2 min

UV: 3x30W lamp, at 80 cm for 12 sec
Conventional heating: 55°C for 5 min

3.9.4.3 Killing effect of different concentration of SDS on *E. coli (lux)*

In a previous experiment, it was shown that SDS 0.1% alone had a small killing effect on the bacterium after 60 min. This experiment was designed to find out the killing effect of various concentrations of SDS (0.01, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5%). The cells were exposed to these concentrations for 5 min at two temperatures, 25 and 50°C. The results are shown in **Tables 3-57 and 3-58**. With concentrations less than 0.2% at 25°C, no little or reduction in viability was observed. At 0.3%, 0.4%, and 0.5% concentrations, 0.11, 0.7 and 0.74 log reductions in the viability were achieved, respectively (**Table 3-57**). At 50°C, much greater reduction in viability was evident with 0.8 - 1.7 log reduction for SDS concentrations greater than 0.2% (**Table 3-58**).

It was concluded that SDS could kill the bacterium in a short time at high concentration, but at lower concentrations it was only effective at higher temperatures. Combination of SDS with other methods e.g. UV, laser and ozone was suggested.

Table 3-57. Effect of different concentrations of SDS on *E. coli (lux)* at 25^0C

NO	Concentration of SDS (%)	Surviving bacteria (cfu/ml)	Log reduction in cfu/ml
1	0	1×10^7	-
2	0.01	1×10^7	0
3	0.05	1×10^7	0
4	0.1	1×10^7	0
5	0.2	9.5×10^6	0.02
6	0.3	7.8×10^6	0.11
7	0.4	2×10^6	0.70
8	0.5	1.8×10^6	0.74

Table 3-58. Effect of different concentrations of SDS on *E. coli (lux)* at 50^0C

NO	Concentration of SDS (%)	Surviving bacteria (cfu/ml)	Log reduction in cfu/ml
1	0	7×10^6	-
2	0.01	6.5×10^6	0.03
3	0.05	6×10^6	0.07
4	0.1	5.7×10^6	0.09
5	0.2	1×10^6	0.84
6	0.3	1×10^6	0.84
7	0.4	9×10^5	0.89
8	0.5	1.5×10^5	1.67

3.9.5 Electron microscopy

Electron microscopy was used to determine any morphological or ultrastructural changes to *E. coli (lux)* induced by the Nd:YAG laser, UV irradiation, conventional heating and combination of the treatments and in different orders. Suspensions of the bacterium were exposed to the different treatments and colony counts were made. Treatment parameters and numbers of surviving bacteria after each treatment are shown below. Samples were prepared using the protocol described in Sections 2.4.5.1 and 2.4.5.2 for scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Treatment	Parameter(s)	Number of surviving bacteria (cfu/ml)	Log reduction (cfu/ml)
Nd:YAG Laser*	9 (sec)	1.5×10^8	0.37
Conventional heating	5 min at 50°C	8×10^7	0.64
UV (3x3W)	10 sec	3×10^7	1.1
L+H+UV	All of above	8.1×10^2	5.63
H+UV+L	All of above	1.5×10^4	4.37
Control	-	3.5×10^8	0

- 20J Power and 15 Hz frequency

SEM findings

With use of this technique, the physical effects of different treatments on the cell surface of *E. coli (lux)* were compared to the control untreated cells. As can be seen in **Figures 3-64 and 3-65,** the surfaces of the untreated cells appeared smooth and unblemished. The cell shapes were regular and many dividing cells and some filaments were observed at low magnification (**Figure 3-64**). **Figures 3-66 and 3-67** show the effect of UV treatment on the surface of the cells. The treatment gave approximately a 1 log reduction in viability (i.e. 90% killing) but the treated cells did not appear significantly different from the control cells. The surfaces were still smooth and some dividing cells were observed. After treatment of the cells with Nd:YAG laser light the cell surfaces changed slightly, **Figures 3-68 and 3-69**. The surfaces had a rough appearance and many curved cells were observed. This was more clearly shown at higher magnification. These changes occurred even though the log reduction in the viability due to the treatment was 0.37. **Figures 3-70 and 3-71** show the effects of conventional heating on the cells. After the treatment, the cell surfaces were slightly damaged and rough. Some misshapen cells were observed, as well as curved cells, as with the laser treatment and some extra cellular debris. The killing rate due to conventional heating was 0.64 log reduction in the viability. The combined treatments gave much greater killing than the sum of the three treatments alone. Also treatment with the order L+H+UV gave better killing (5.63 log) than the order H+UV+L (4.37 log) as observed previously, but no significant differences were observed in the cell surfaces between the two different orders. Results for the order L+H+UV are shown in **Figures 3-72 and 3-73** and for the order H+UV+L in **Figures 3-74 and 3-75**. The damage to the cells was a little more prominent after the treatment with the combined methods. The cell surfaces after both treatments appeared shrivelled and roughened and more misshapen, some extra cellular debris and curved cells were observed. This was more apparent at higher magnification (**Figure 3-75**). Few dividing cells were observed after treatment of the cells with both combined treatments.

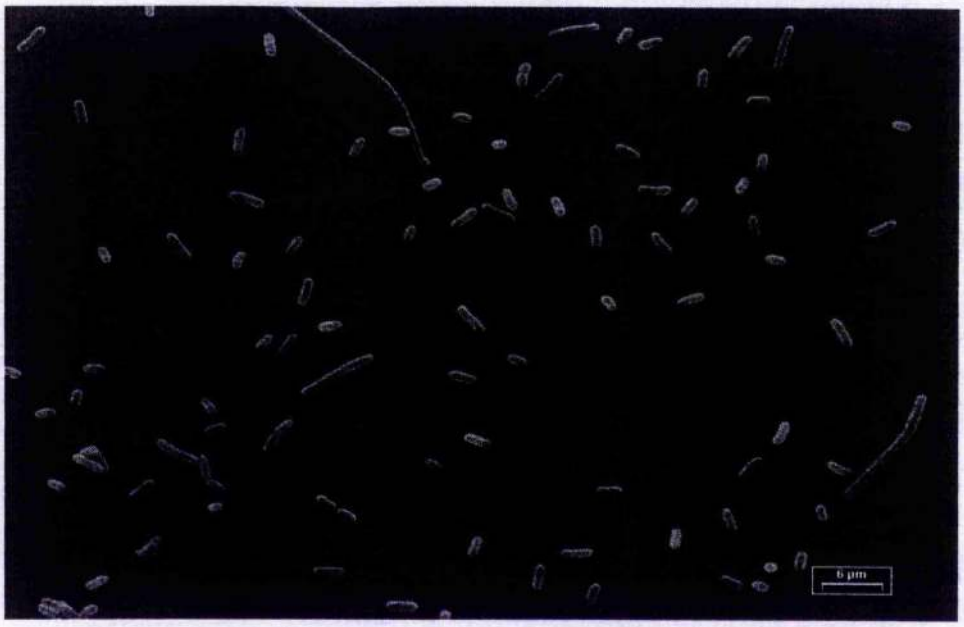


Figure 3-64. Scanning electron micrograph of *E. coli (lux)*, control preparation

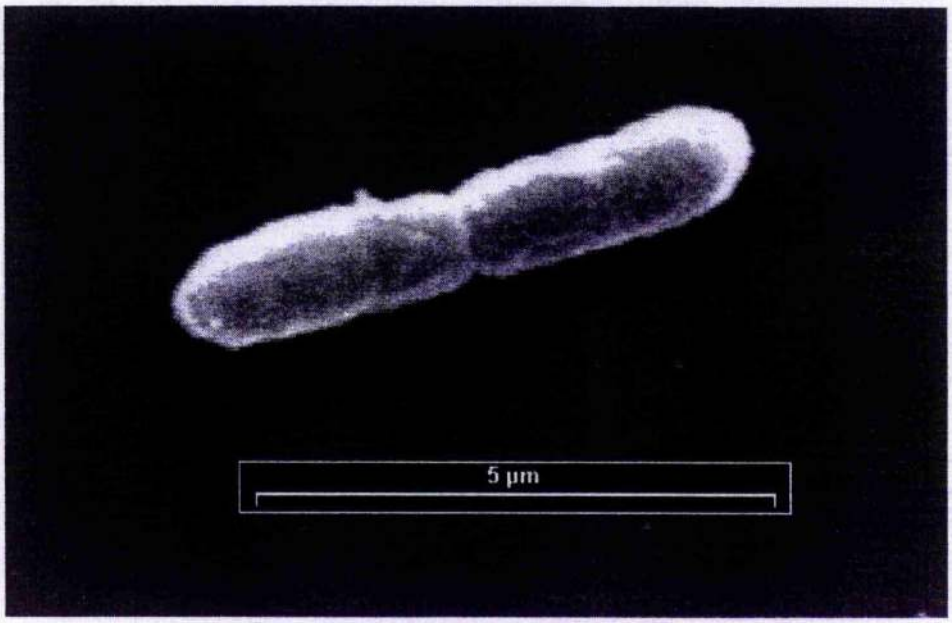


Figure 3-65. Scanning electron micrograph of *E. coli (lux)*, control preparation

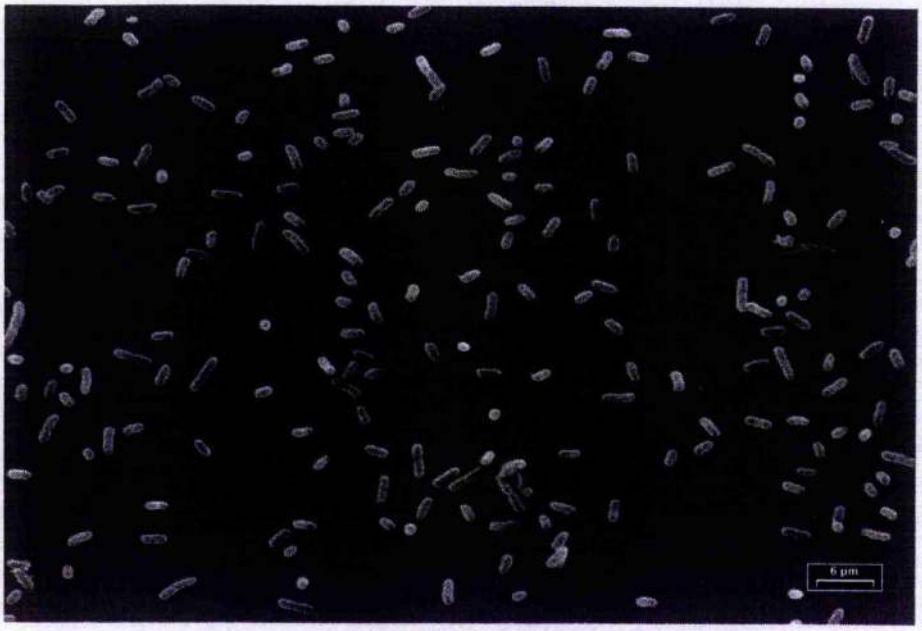


Figure 3-66. Scanning electron micrograph of *E. coli (lux)*, after UV treatment

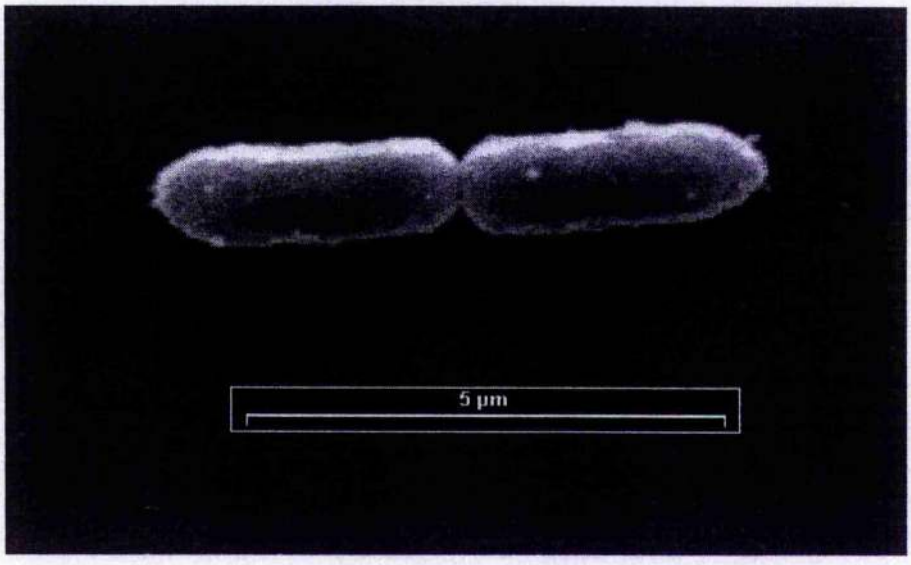


Figure 3-67. Scanning electron micrograph of *E. coli (lux)*, after UV treatment

Figure 3-68. Scanning electron micrograph of *E. coli (lux)*, after Nd:YAG laser treatment

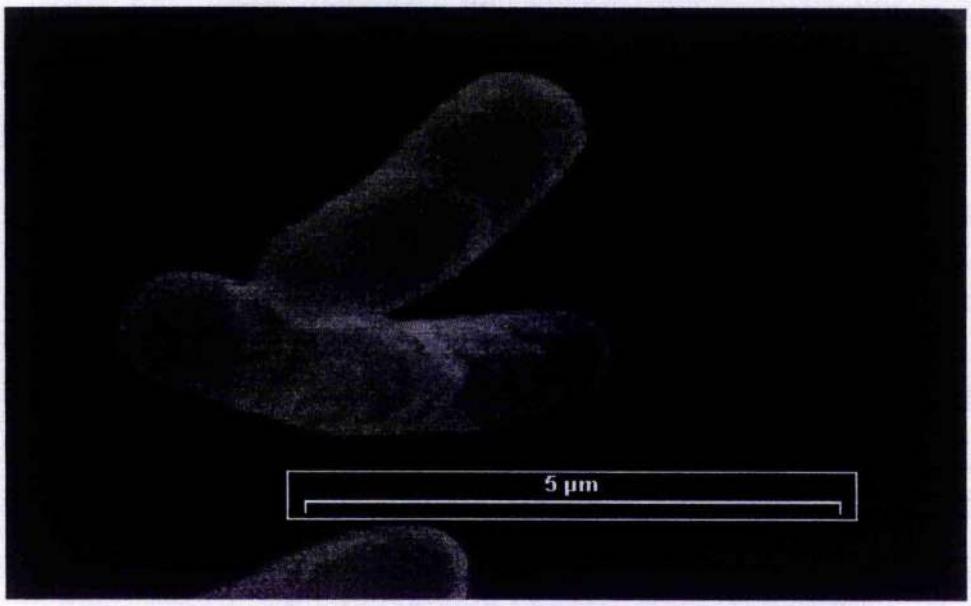


Figure 3-69. Scanning electron micrograph of *E. coli (lux)*, after Nd:YAG laser treatment

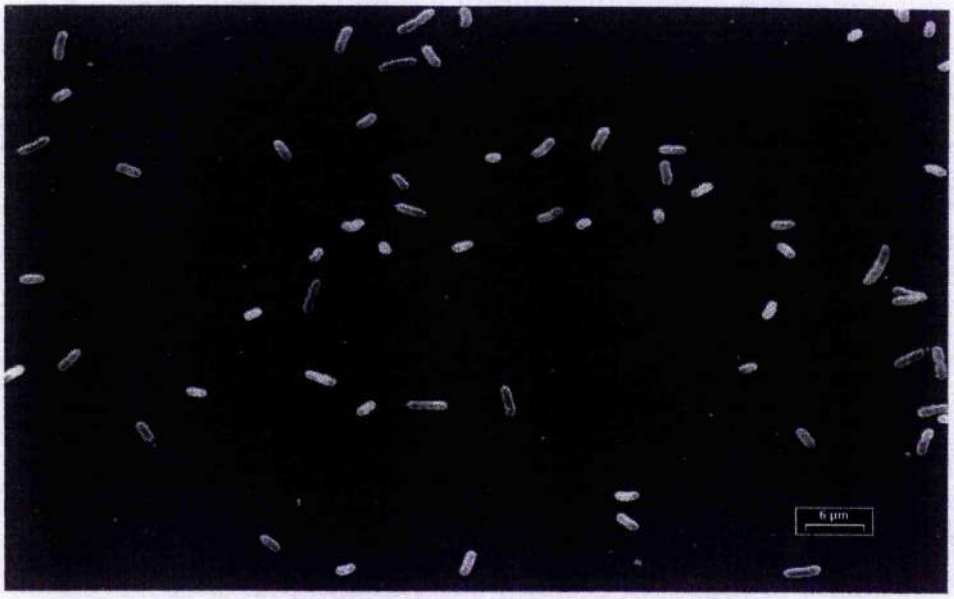


Figure 3-70. Scanning electron micrograph of *E. coli (lux)*, after conventional heating treatment

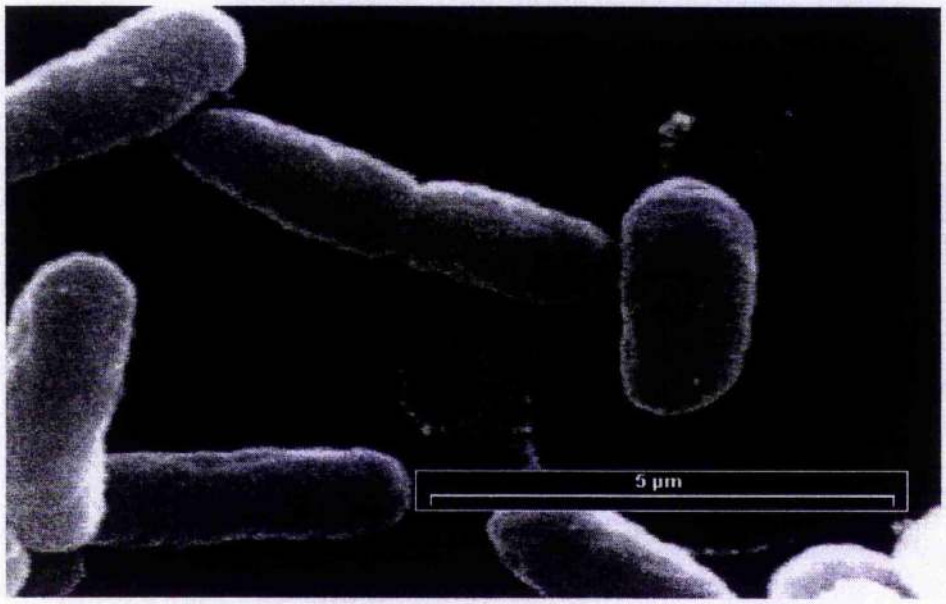


Figure 3-71. Scanning electron micrograph of *E. coli (lux)*, after conventional heating treatment

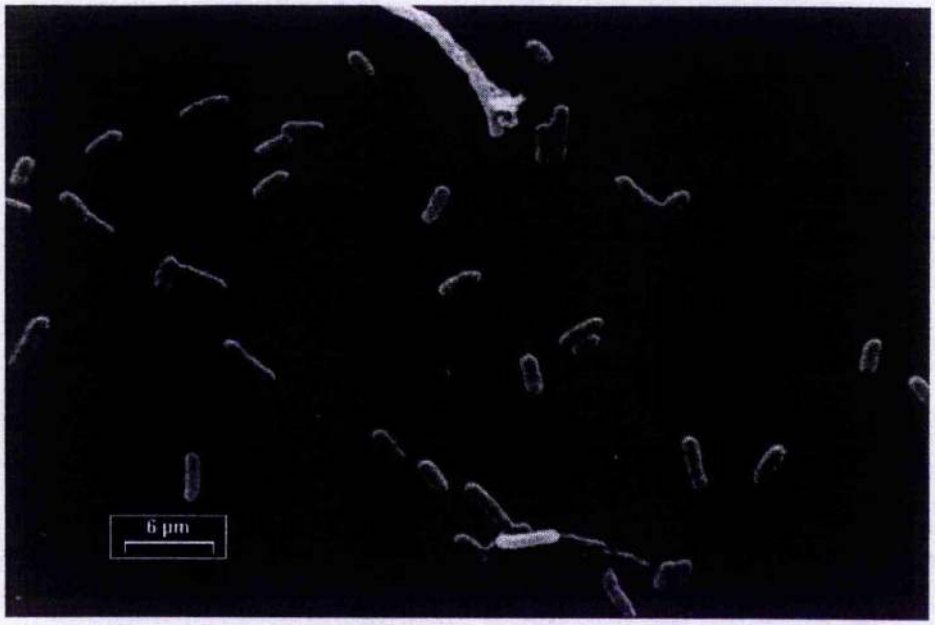


Figure 3-72. Scanning electron micrograph of *E. coli (lux)*, after sequential treatment with the order, laser, conventional heating then UV

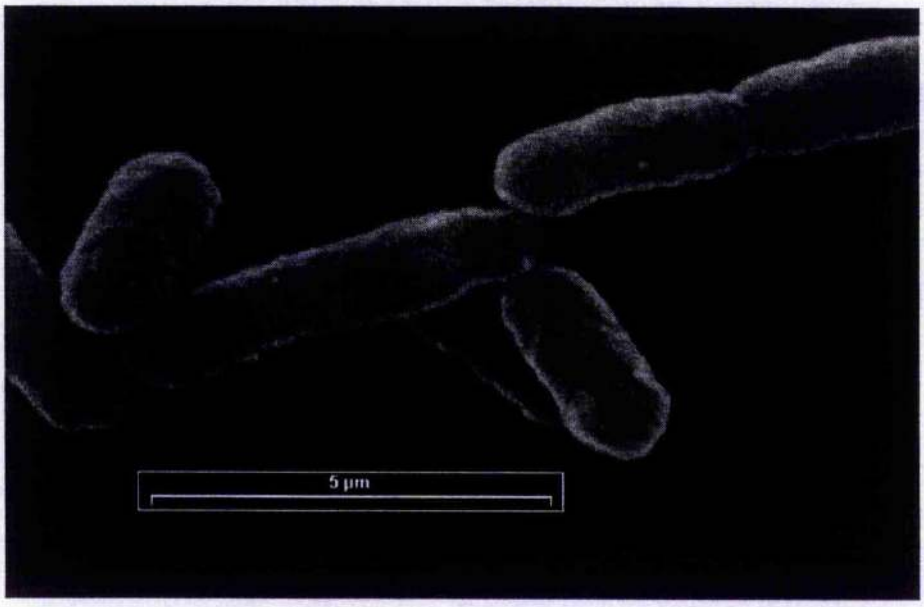


Figure 3-73. Scanning electron micrograph of *E. coli (lux)*, after sequential treatment with the order, laser, conventional heating then UV

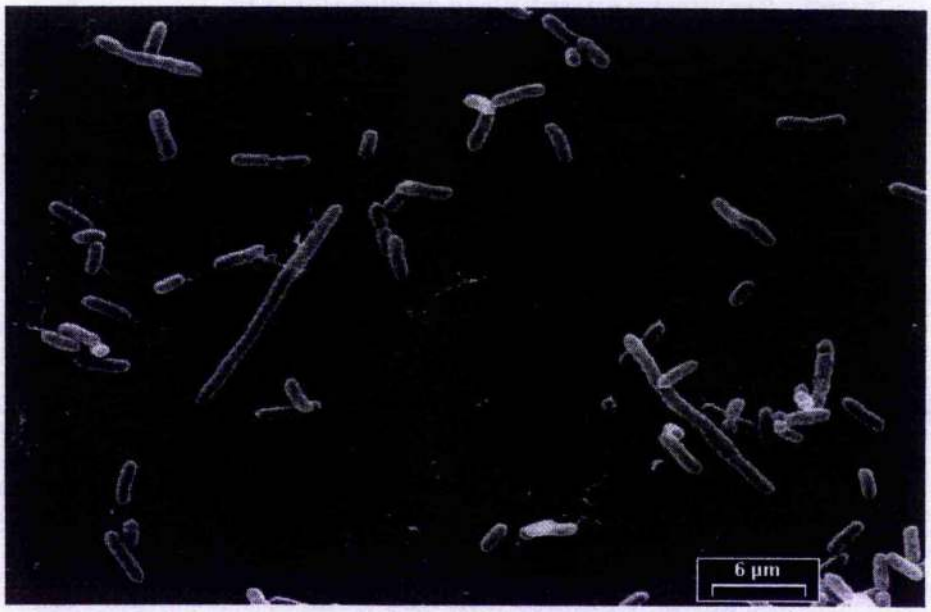


Figure 3-74. Scanning electron micrograph of *E. coli (lux)*, after sequential treatment with the order, conventional heating, UV then laser

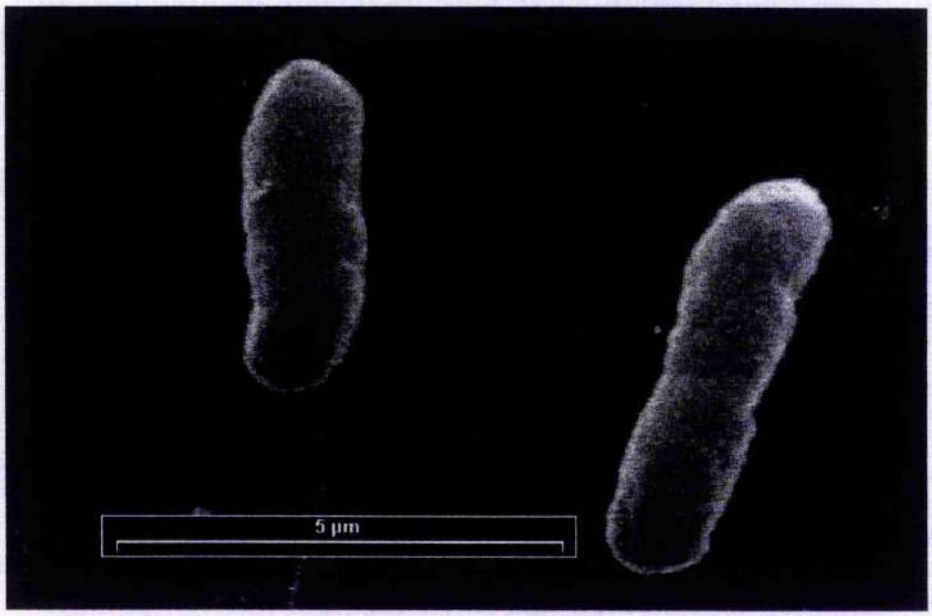


Figure 3-75. Scanning electron micrograph of *E. coli (lux)*, after sequential treatment with the order, conventional heating, UV then laser

TEM findings

With use of the TEM, the physical effects of different treatments on the internal structure of the *E. coli (lux)* could be compared to those in the control untreated cells. Thin sections of the untreated cells can be seen in **Figures 3-76 and 3-77**. The cell envelopes are intact and no abnormality was observed in the organisation of the cell contents, with clear diffuse nuclear areas and dense cytoplasm observed. **Figures 3-78 and 3-79** show cells treated with UV. No significant abnormalities were observed in the cells. **Figures 3-80 and 3-81** show the effect of laser irradiation on the cells. As can be seen at the low magnification, many vacuoles or less dense areas appeared in the cytoplasm as a result of the treatment. There also appeared to be amorphous material surrounding the cells, as if it had been released from damaged cells. Many cells in **Figure 3-80** show large periplasmic areas at the poles of the cells, but such areas were only seen occasionally in the control cells (**Figure 3-77**). The cells treated with conventional heating are shown in **Figures 3-82 and 3-83**. The cells look normal and similar to the untreated cells. Rarely, vacuoles or less dense areas were observed in some cells (**Figure 3-83**). **Figures 3-84 and 3-85** show treated cells with the order L+H+UV and **Figures 3-86 and 3-87** show the treated cells with the order H+UV+L. The morphological changes are not as obvious as when the cells were treated by laser alone but the nuclei appear more condensed.

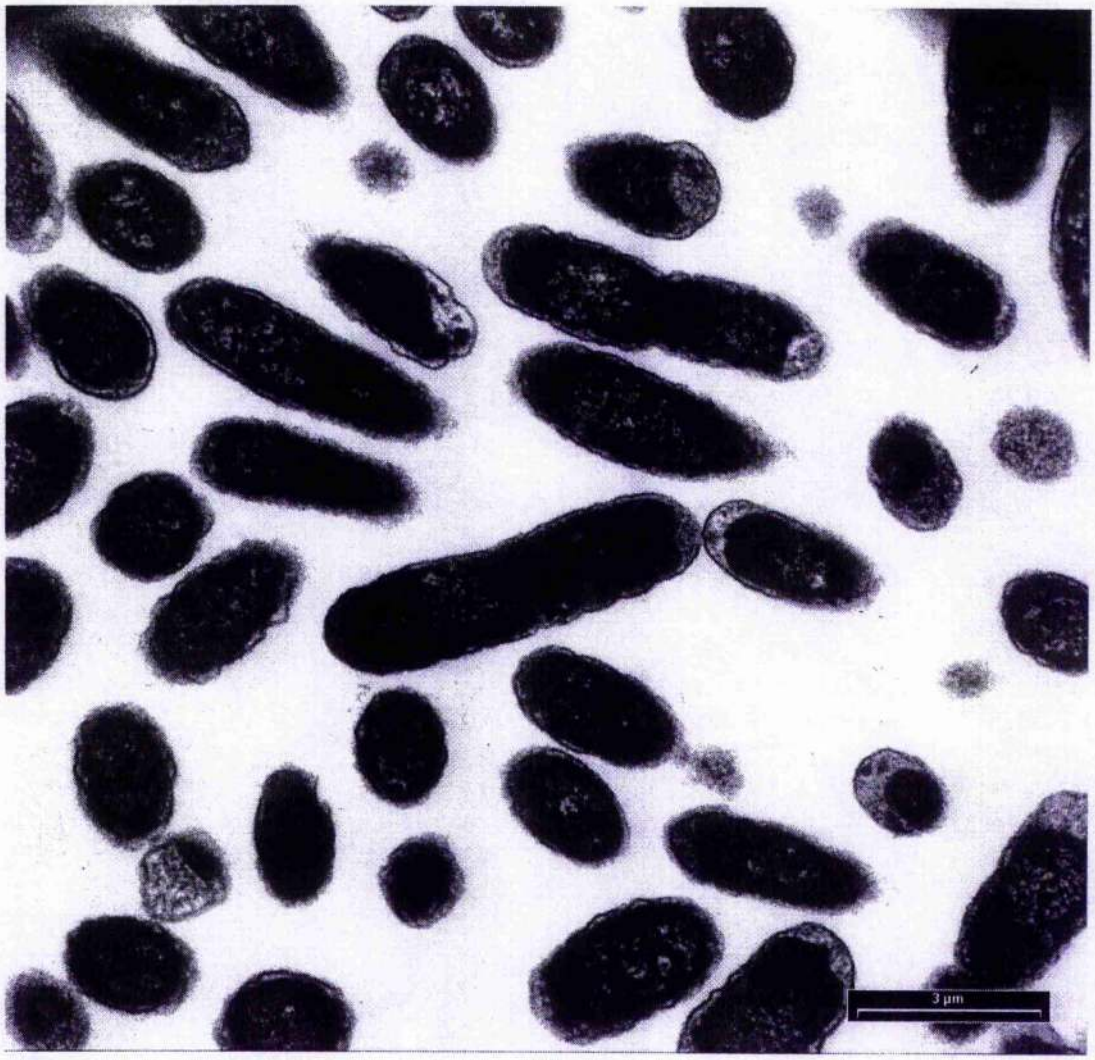


Figure 3-76. Transmission electron micrograph of ultra thin sections of *E. coli (lux)*, control preparation

Figure 3-77. Transmission electron micrograph of ultra thin sections of *E. coli (lux)*, control preparation

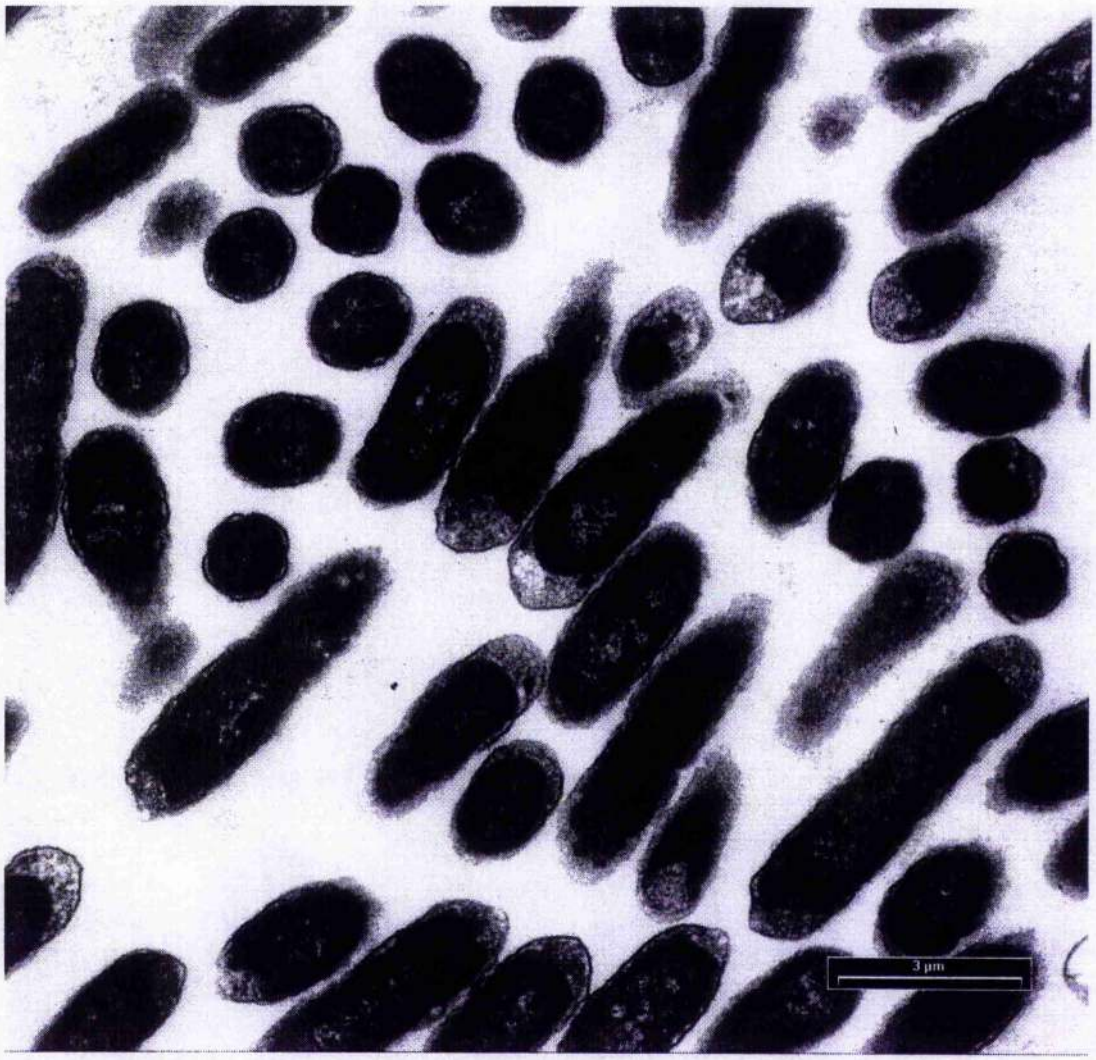


Figure 3-78. Transmission electron micrograph of ultra thin sections of *E. coli (lux)*, after UV treatment

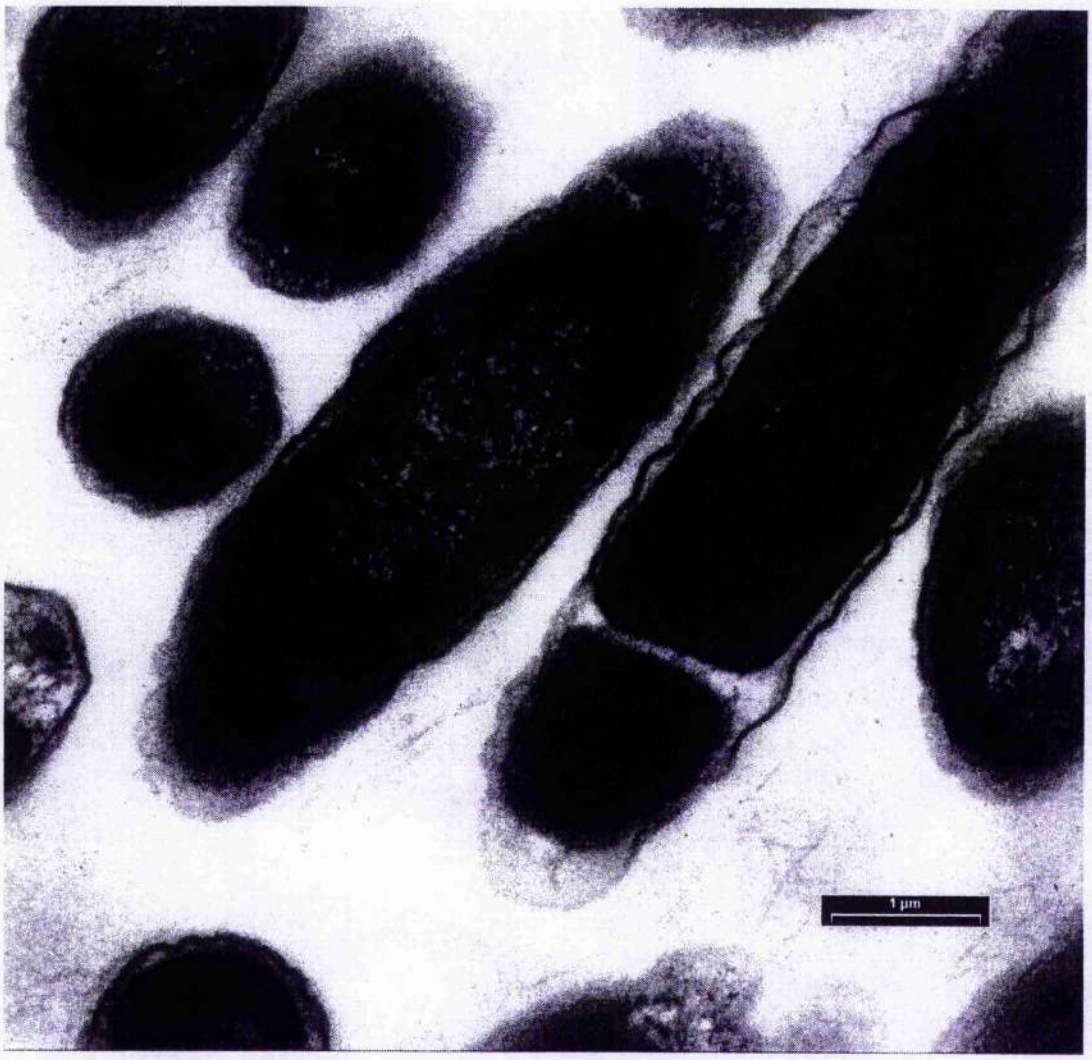


Figure 3-79. Transmission electron micrograph of ultra thin sections of *E. coli (lux)*, after UV treatment

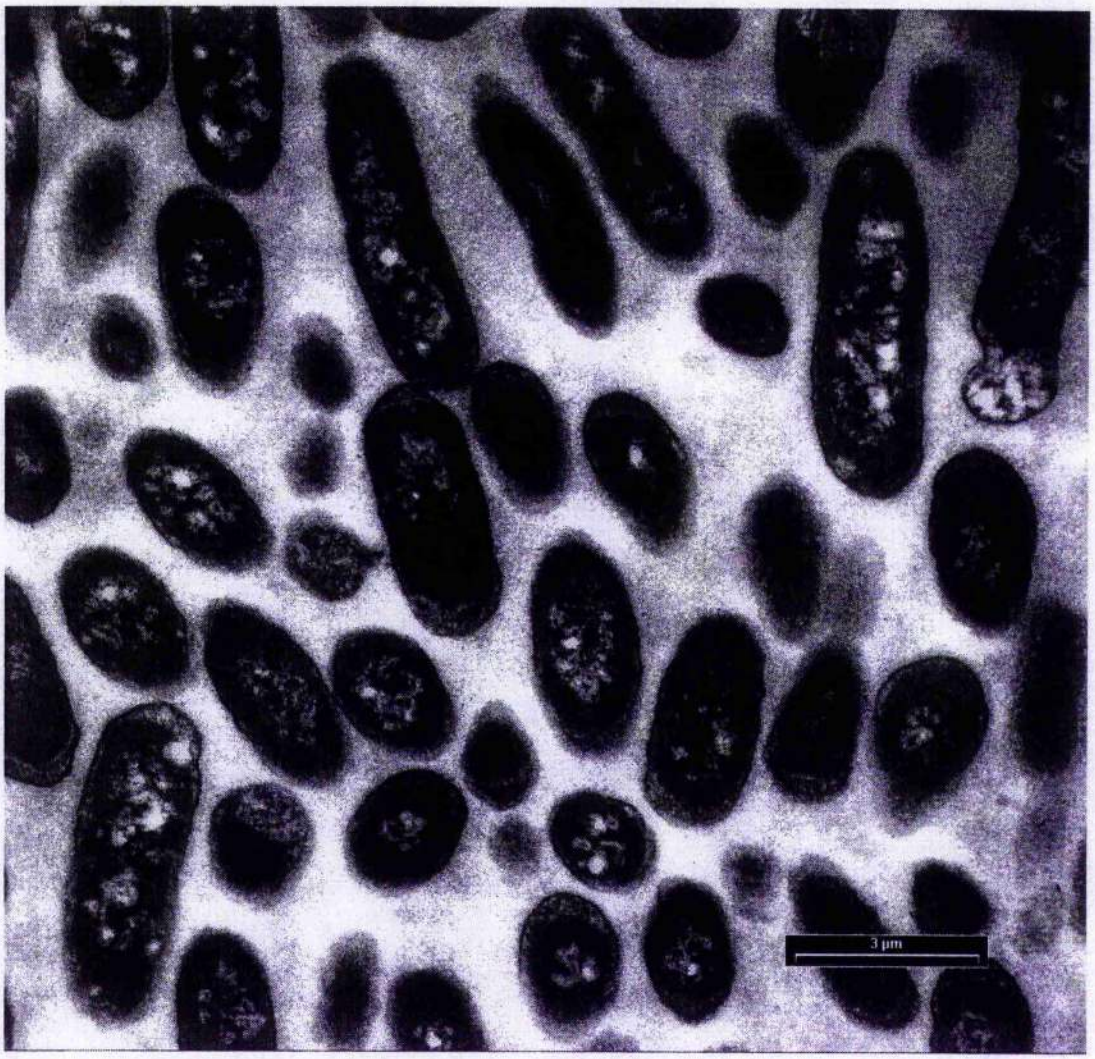


Figure 3-80. Transmission electron micrograph of ultra thin sections of *E. coli (lux)*, after laser treatment

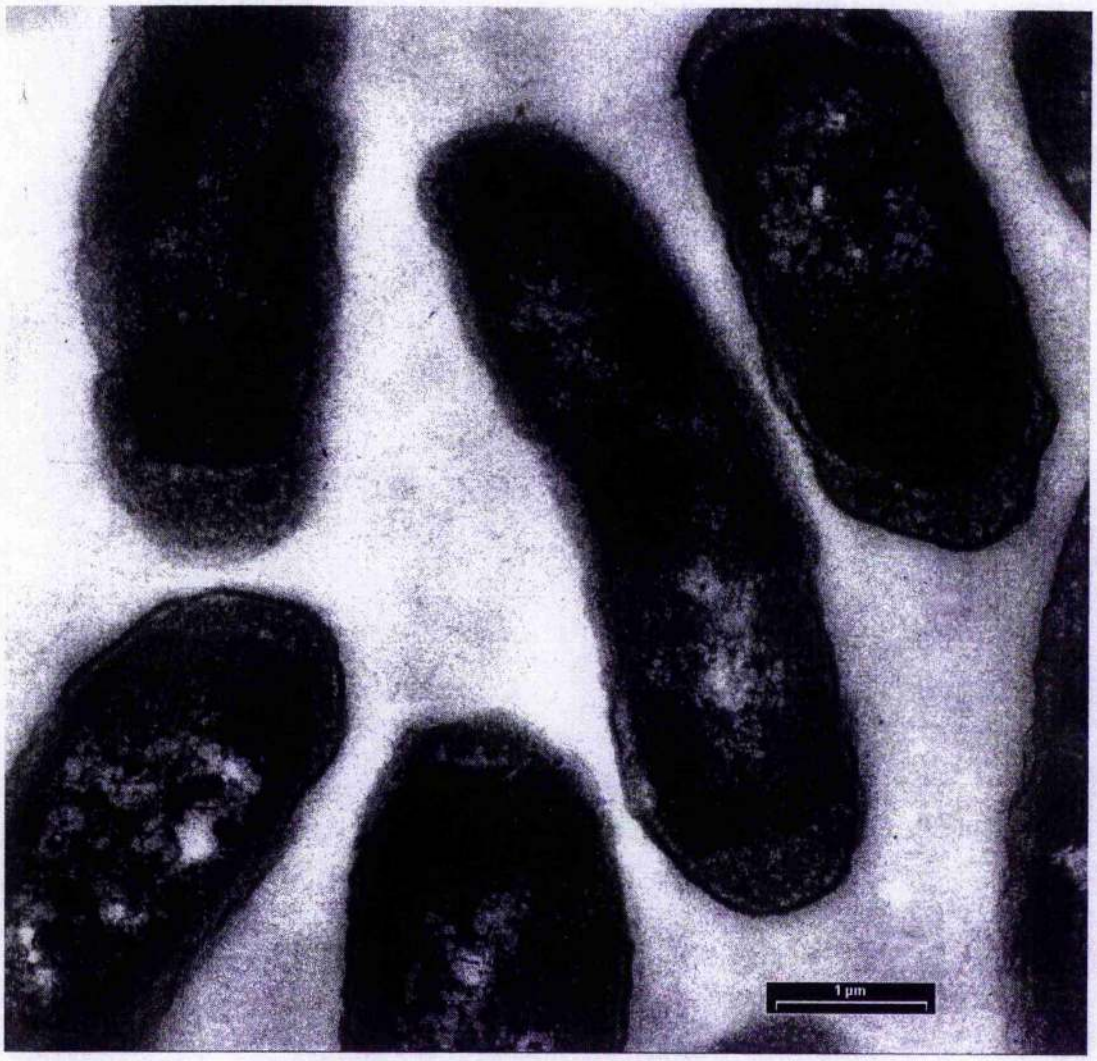


Figure 3-81. Transmission electron micrograph of ultra thin sections of *E. coli (lux)*, after laser treatment

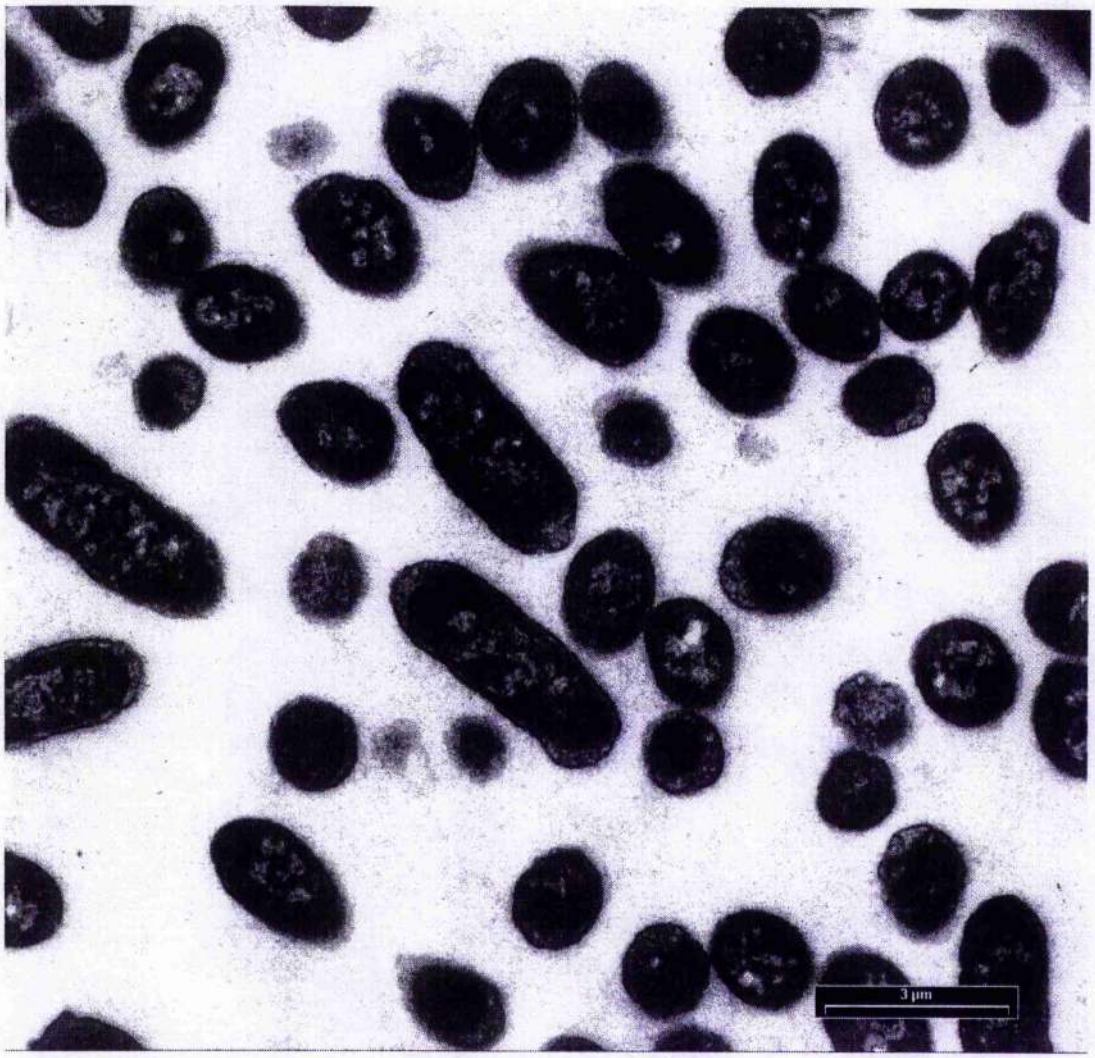


Figure 3-82. Transmission electron micrograph of ultra thin sections of *E. coli (lux)*, after conventional heating treatment

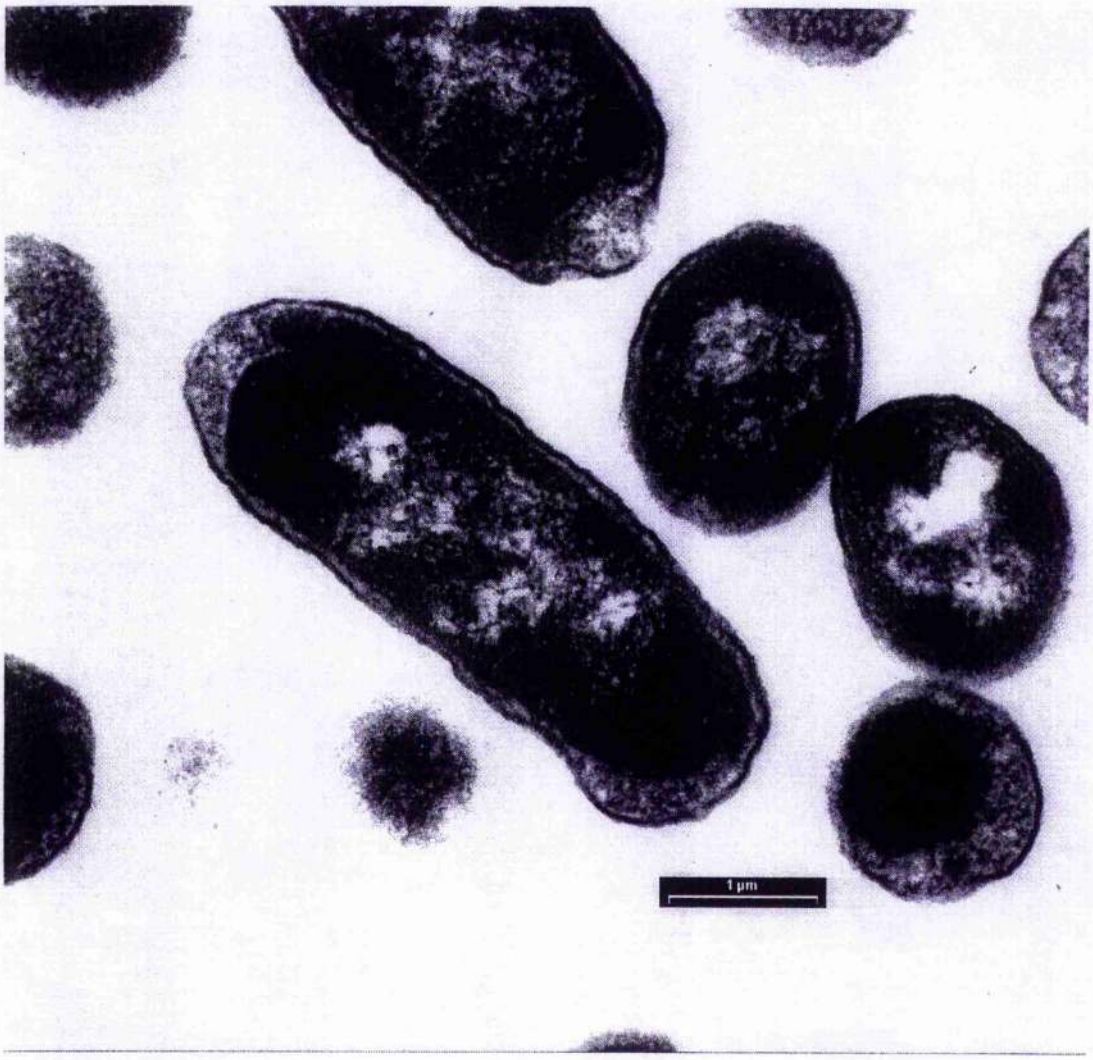


Figure 3-83. Transmission electron micrograph of ultra thin sections of *E. coli (lux)*, after conventional heating treatment

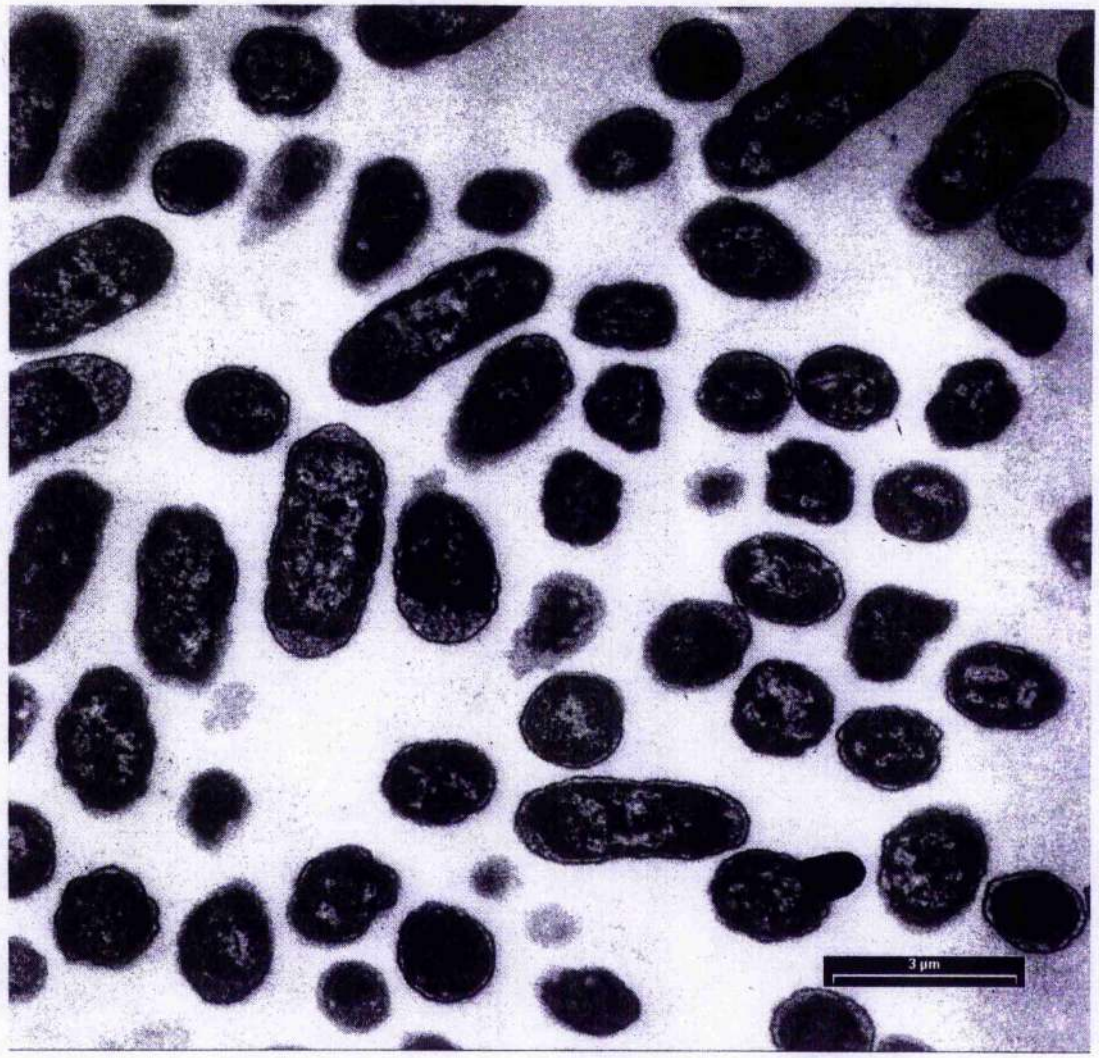


Figure 3-84. Transmission electron micrograph of ultra thin sections of *E. coli (lux)*, after sequential treatment with the order, laser, conventional heating then UV

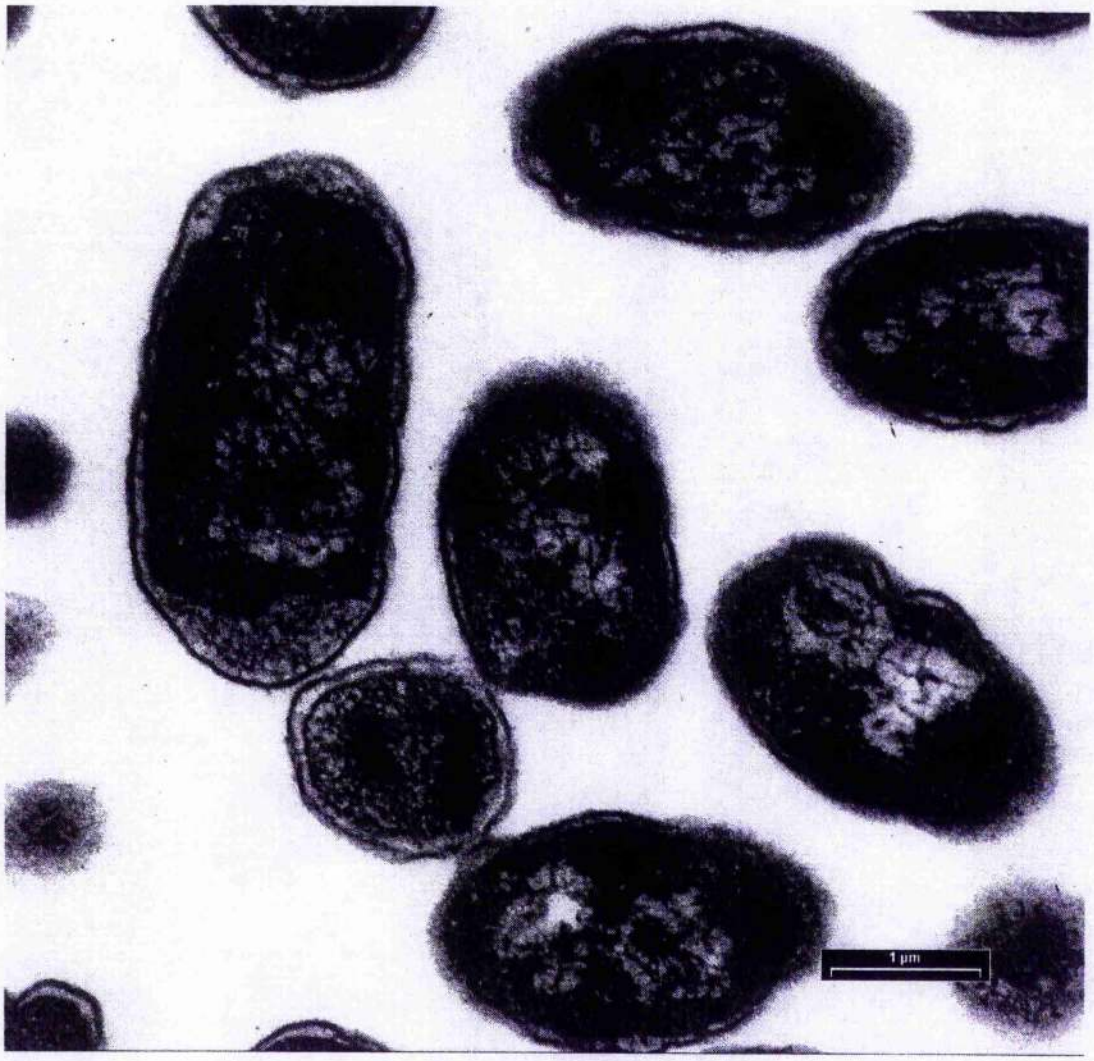


Figure 3-85. Transmission electron micrograph of ultra thin sections of *E. coli (lux)*, after sequential treatment with the order, laser, conventional heating then UV

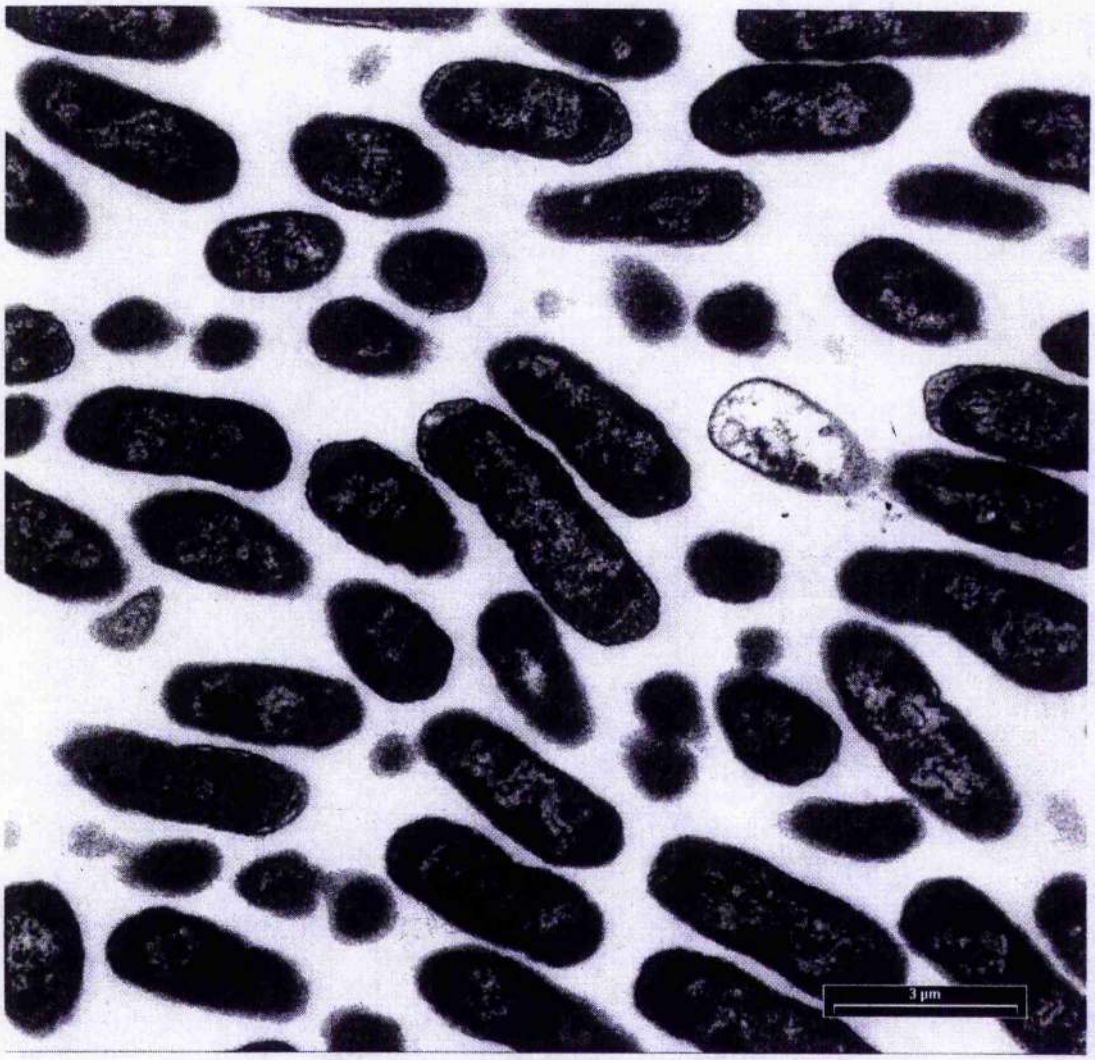


Figure 3-86. Transmission electron micrograph of ultra thin sections of *E. coli (lux)*, after sequential treatment with the order, conventional heating, UV then laser

Figure 3-87. Transmission electron micrograph of ultra thin sections of *E. coli (lux)*, after sequential treatment with the order, conventional heating, UV then laser

3.10 Decontamination of selected foodstuffs

For investigation of the practical aspects of killing bacteria by different treatments and combination methods on actual food samples smoked salmon was selected. This product is prepared in thin slices with a firm, smooth surface and was considered as a suitable material. First, a general bacteriological examination of various seafood samples, including smoked salmon, obtained from local shops was done to investigate the total bacterial counts and to isolate and enumerate Listeria spp.

3.10.1 Total count and *Listeria* count of seafood

In total, 24 samples were investigated. These included: seventeen samples of chilled smoked salmon, 3 samples of cooked prawns, 2 samples of salmon fillet, 1 sample of frozen smoked salmon and 1 sample of smoked rainbow trout. In general, the numbers of aerobic bacteria usually found on seafood products such as these is high and the values up to 10^6-10^7 cfu/cm are typical (172). Results are shown in **Table 3-59** and **Table 3-60.** Total bacterial counts at different temperatures showed, except for few samples, that the levels of aerobic organisms were normal. The means (and ranges) of the total aerobic counts in all samples at 18°C was $5.1x10^5$ ($1x10^3$ to $1x10^7$), at 30°C was $1x10^6$ ($1x10^3$ to $2 x10^7$) and at 37°C was $3.2x10^5$($1x10^3$ to $5x10^6$). In one sample of salmon fillet and one of chilled smoked salmon, the bacterial levels were much higher than the average. These samples were approaching their sell-by date when they were purchased and examined.

Two samples of smoked salmon were found to contain *L. monocytogenes.* The bacteria were isolated after enrichment for 24h at 30°C in Listeria enrichment broth and then subcultured on Oxford agar at 37°C for 24h, as described in section 2.5.2. The colonies after purification were confirmed by Gram staining and the API®Listeria system. None of the colonies that were isolated by direct plating of the homogenized samples on these media were confirmed as *L. monocytogenes* and it was not possible to obtain direct counts for the pathogens. 15 unknown Gram-positive bacteria were grown on the Oxford agar and Listeria monocytogenes blood agar (LMBA) that the API tests showed were not related to Listeria. Also, 4 unknown Gram-negative bacteria were isolated on the Oxford agar and LMBA.

Table 3-59. Bacteriological examination of seafood

Type	Total counts at 18°C* cfu/g	Total counts at 30°C cfu/g	Total counts at 37°C cfu/g	Suspected *Listeria* on Oxford agar	Suspected *Listeria* on LMBA	Growth of unknown bacteria on Oxford agar	Growth of unknown bacteria on LMBA
Frozen smoked salmon	2.5 x 103	2 x 103	3.1 x 103	-	-	+	-
Chilled smoked salmon	2 x 103	4 x 103	1 x 103	-	-	+	+
Chilled smoked salmon	2.4×10^4	1×10^3	1.2×10^3	+	+	-	-
Chilled smoked salmon	5×10^3	6.5×10^4	6×10^4	-	-	-	-
Chilled smoked salmon	1.2×10^5	1.3×10^5	8×10^4	-	-	+	+
Cooked prawns	5×10^3	1.8×10^3	-/?	-	-	+	+
Smoked rainbow trout	4.3×10^5	8.5×10^5	3.6×10^5	+	-	-	+
Salmon fillet	7.7×10^4	8×10^5	3×10^4	+	-	-	+
Chilled smoked salmon	2.6×10^4	2.4×10^4	1.5×10^4	-	-	-	-
Cooked prawns	7.1×10^5	1.4×10^5	2.3×10^4	-	-	-	-
Chilled smoked salmon	1×10^3	1.5×10^3	5×10^2	-	-	+	+
Salmon fillet	1×10^7	2×10^7	5×10^6	+	-	+	+
Chilled smoked salmon	4×10^9	5.2×10^8	7.9×10^5	-	-	+	+
Cooked prawns	1.5×10^4	2×10^4	4×10^3	-	-	-	-
Chilled smoked salmon	7×10^2	9×10^2	2×10^2	-	-	-	-

Continued over

Bacterial examination of seafood (continued)

Type	Total counts at 18°C cfu/g	Total counts at 30°C cfu/g	Total counts at 37°C cfu/g	Suspected *Listeria* on Oxford agar	Suspected *Listeria* on LMBA	Growth of unknown bacteria on Oxford agar	Growth of unknown bacteria on LMBA
Chilled smoked salmon	4×10^4	4.5×10^4	3×10^5	+	+	-	-
Chilled smoked salmon	2.8×10^3	4.3×10^3	3×10^3	-	-	+	-
Chilled smoked salmon	1.5×10^4	2×10^4	4×10^3	-	-	+	+
Chilled smoked salmon	2.1×10^3	3×10^3	5.6×10^2	+**	-	-	-
Chilled smoked salmon	5×10^3	6.5×10^4	6×10^4	-	-	-	-
Chilled smoked salmon	1.5×10^5	1.3×10^6	9.5×10^5	+**	-	-	-
Chilled smoked salmon	5×10^3	1.5×10^4	2.2×10^4	-	-	-	-
Chilled smoked salmon	1.1×10^3	$8x\ 10^3$	4.2×10^4	-	-	-	-
Chilled smoked salmon	6.5×10^3	5×10^4	1.5×10^4	-	-	-	-

* Incubation temperature

** *Listeria monocytogenes* was confirmed

Table 3-60. Summary of bacterial examination of seafood

Sample	No	Mean of total count at 18°C cfu/g	Mean of total count at 30°C cfu/g	Mean of total count at 37°C cfu/g	Isolation of *L. monocytogenes*	Isolation of other *Listeria*	Isolation by Oxford agar	Isolation by LMBA
Chilled smoked salmon	17	2.5×10^4	1.1×10^5	9.7×10^4	2	0	2	0
Cooked prawns	3	2.4×10^5	5.4×10^4	1.3×10^4	0	0	0	0
Salmon fillet	2	5×10^6	1.1×10^7	2.5×10^6	0	0	0	0
Frozen smoked salmon	1	2.5×10^3	2×10^3	3.1×10^3	0	0	0	0
Smoked rainbow trout	1	4.3×10^5	8.5×10^5	3.6×10^5	0	0	0	0
Total	24	5.1 x 105	1 x 106	3.2 x 105	2	0	2	0

3.10.2 Investigation of decontamination of smoked salmon by combination of conventional heating, UV and laser

3.10.2.1 Killing of *L. monocytogenes* on smoked salmon

Smoke salmon samples were inoculated with a suspension of *L. monocytogenes* as described in section 2.5.2. The samples were then exposed to different treatments and in different orders. Listeria enrichment broth, Oxford agar and LMBA were used to enumerate *L. monocytogenes* using protocol as described in Section 2.5.2. Before inoculation of the sample, the total counts on Plate count agar and a Listeria count were made to ensure that it was *Listeria*-free. The treatments used separately are shown below:

CO_2 laser: 100 W, 30, 60,90 and 120 ms exposure time
UV (3x30W): 6,8,10 and 15 sec at 80 cm
Conventional heating: incubator, 5 min 50°C

For combination of treatments, the following parameters were used:

Laser: 60 ms, 100 W
UV: 15 sec, 80 cm
Heat: 50°C, 5 min

The experiment was done twice and the mean of the results are shown in **Table 3-61** for separate treatments and **Table 3-62** for the combination of three treatments in two different orders. The bacterial counts of the samples are shown in **Table 3-63**. The samples were *Listeria*-free and the mean of total counts at 18°C was 7×10^3, at 30°C was 4.5×10^5 and at 37°C was 4.2×10^5. Except for conventional heating, all treatments alone gave similar log reductions in viability (0.25-0.35 logs) in the *Listeria* counts on the smoked salmon surface. There was no obvious difference in killing between laser treatment for 30, 60 or 90 ms or between UV for 8 or 10 sec. UV for 15 sec however gave a slightly greater effect. Heating at 50°C for 5 min had little effect on the *Listeria* counts with a log reduction of 0.16 (**Table 3-61**). When sequential treatments were used

in the order, L + H + UV, a total log reduction in viability of 0.93 was observed, whereas the order H + UV + L gave a log reduction of 0.61.

Table 3-61. Killing effect of individual treatments on *L. monocytogenes* in smoked salmon

	Treatment	Mean of surviving bacteria (cfu/g)	Mean of Log reduction in cfu/g	Log reduction STDEV*
1	Control	1.1×10^5	-	-
2	30 ms laser	6.2×10^4	0.25	0.09
3	60 ms laser	6.1×10^4	0.26	0.01
4	90 ms laser	5.6×10^4	0.29	0.06
5	8 sec UV	6×10^4	0.26	0.10
6	10 sec UV	6×10^4	0.26	0.05
7	15 sec UV	4.7×10^4	0.36	0.03
8	Heating 50°C	7.5×10^4	0.16	0.12

* Standard deviation

Table 3-62. Killing effect of combination of treatments on *L. monocytogenes* on smoked salmon

	Treatment	Mean of surviving bacteria (cfu/g)	Mean of Log reduction in cfu/g	Log reduction STDEV
1	Control	2×10^5	-	-
2	L + H + UV	2.3×10^4	0.93	0.28
3	H + UV + L	4.8×10^4	0.61	0.11
Sum of 3 treatments alone*			0.78	

* Data from Table 3-61

Table 3-63. Total bacterial count in smoked salmon samples used for treatments

	Mean of enumerated cells (cfu/g)
Total count (18°C)	7×10^3
Total count (30°C)	4.5×10^5
Total count (37°C)	4.2×10^5
Listeria count	Negative

The sum of the log reductions caused by the three treatments applied individually was approximately 0.75 log. Thus, there may be a small synergistic effect of the three treatments on the killing of *L. monocytogenes* on smoked salmon by the order L + H + UV. In the treatment by CO_2 laser, some discoloration and burning was observed on the surface of the smoked salmon after exposure for 60 ms and longer.

3.10.2.2 Killing of *E. coli (lux)* on smoked salmon

This experiment was done to determine the killing effect of different treatments on *E. coli (lux)* on the surface of smoked salmon. The parameters used are shown below:

Laser: CO_2 laser 60 ms, 100 W
UV: 10 sec, 80 cm
Heat: 50°C, 8 min

The same procedure was used as described in Section 2.5.3. Nutrient agar containing ampicillin was used for the recovery and enumeration of the bacterium from the samples. No colonies were isolated from the samples on the medium before inoculation; the total counts found are shown in **Table 3-63**. Results of the treatments are shown in **Table 3-64**. As in the previous experiment, little reduction in bacterial counts was apparent after the individual treatments alone. Also, the killing effect of the combination treatments with the order, L + H + UV, was similar (0.54 log reduction in viable count) to the sum of the killing effect of the treatments alone. The killing effect of the order H + UV + L was even smaller (0.38 log reduction). Based on these results, it was concluded that probably most bacteria were hidden to the treatment by the rough surface of the salmon flesh and were not exposed to the electromagnetic treatments.

3.10.3 Decontamination of *L. monocytogenes* on smoked salmon by ozone

Ozone gas was used to investigate decontamination of smoked salmon inoculated with *L. monocytogenes*. The protocol is described in Section 2.5.4. A modified agar was found useful to recover the highest number of stressed Listeria cells from the sample. Results are shown in **Figs 3-88** and **3-89** and the effect of ozone at different places of treatment chamber can be seen. After exposure of salmon samples to ozone for 10 min, log reductions in *Listeria* count of 0.16, 0.72, 0.62 and 0.51 was achieved for positions Q1 to Q4, respectively (see **Figure 2-2**). After 15 min, these values were increased to 0.87, 0.82, 0.50 and 0.93, respectively.

Table 3-64. Killing effect of different treatments on *E. coli (lux)* on smoked salmon

	Treatment	Mean of surviving bacteria (cfu/g)	Mean of log reduction in cfu/g	Log reduction STDEV
1	**Control**	**3.5×10^5**	-	-
2	**UV**	**2.2×10^5**	**0.21**	0.04
3	**Laser**	**2.3×10^5**	**0.19**	0.07
4	**Heat**	**2.5×10^5**	**0.14**	0.12
5	**L + H + UV**	**1×10^5**	**0.54**	0.24
6	**H + UV + L**	**1.5×10^5**	**0.38**	0.14
Sum of 3 treatments alone			**0.54**	

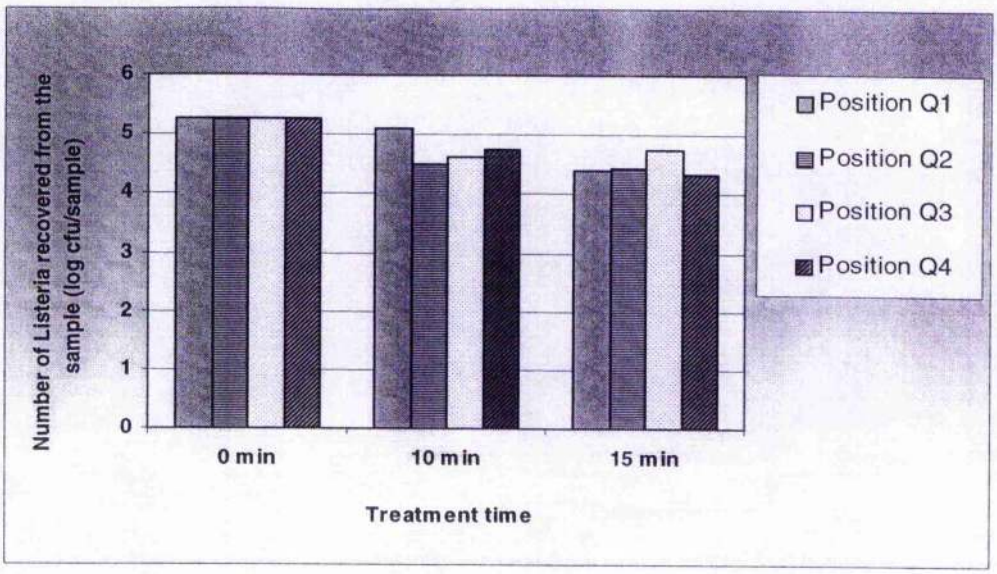


Figure 3-88. Killing of *L. monocytogenes* on smoked salmon by ozone

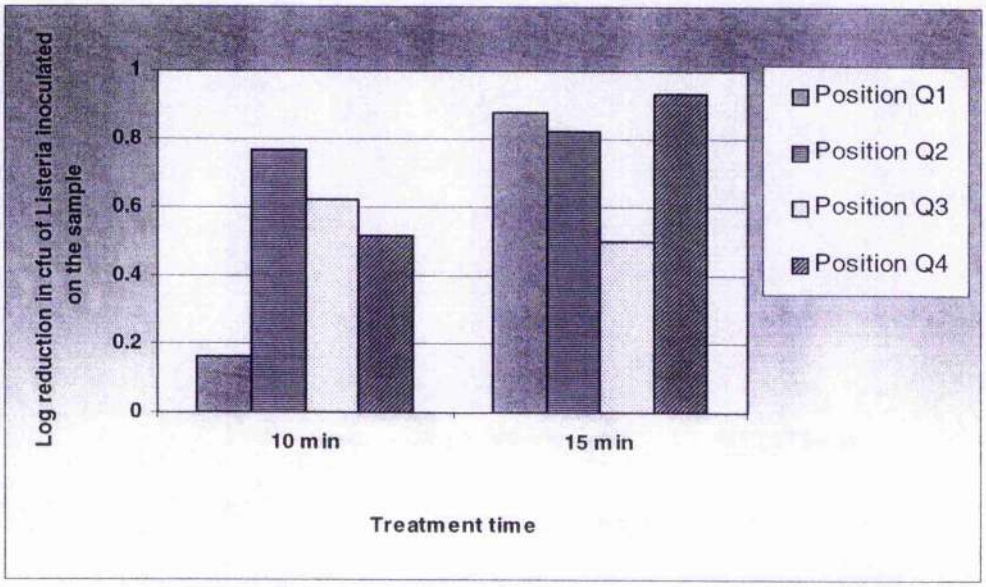


Figure 3-89. Reduction by ozone of the viability of *L. monocytogenes* on smoked salmon

3.10.4 Decontamination of selected bacteria on chicken skin by ozone

In this experiment, the effect of ozone was investigated for decontamination of *S. typhimurium, S. aureus, C. jejuni* and *L. monocytogenes* applied to the surface of chicken skin. As described in Section 2.5.5, chicken skin samples were inoculated with suspensions of the different bacteria, treated for 5, 10 and 15 min. with ozone and surviving cells were recovered on the appropriate selective agars. Counts from untreated samples were done on the same medium to serve as the controls. Results are shown in **Figures 3-90** for *S. typhimurium*, **3-92** for *S. aureus*, **3-94** for *C. jejuni* and **3-96** for *L. monocytogenes*. The log reductions achieved by each treatment for each bacterium are shown in **Figures 3-91, 3-93, 3-95** and **3-97** for *S. typhimurium, S. aureus, C. jejuni* and *L. monocytogenes,* respectively. Different results were obtained for different samples treated in different positions in the chamber (Q1 to Q4). After treatment for 10 min, about 0.3 log reduction in viable count was achieved for *S. typhimurium*, whereas the values were 0.2, 0.8-1.0 and 0.6 logs for *S. aureus, C. jejuni* and *L. monocytogenes*, respectively. Treatment of samples with ozone for 15 min. gave greater killing, with 0.4, 0.4-0.8, 0.7-1.2 and 0.9 log reduction in viable counts for *S. typhimurium, S. aureus, C. jejuni* and *L. monocytogenes*, respectively. Although the best killing results was obtained for *C. jejuni* after 15 min ozonation, it should be born in mind that the experiment was done once and also there was a large difference between the four positions of the plates.

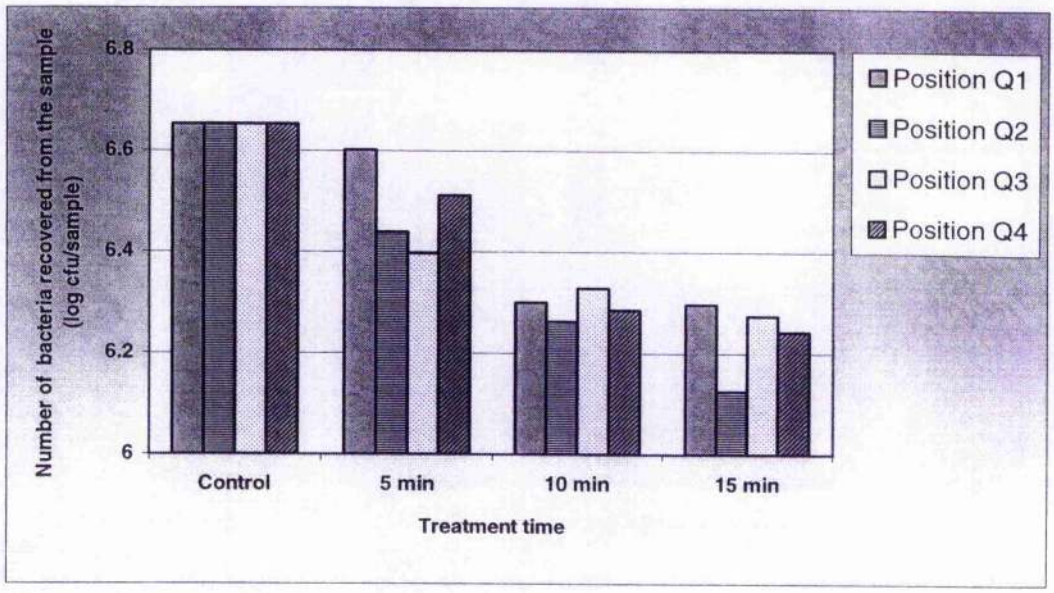


Figure 3-90. Killing of *S. typhimurium* on chicken skin by ozone

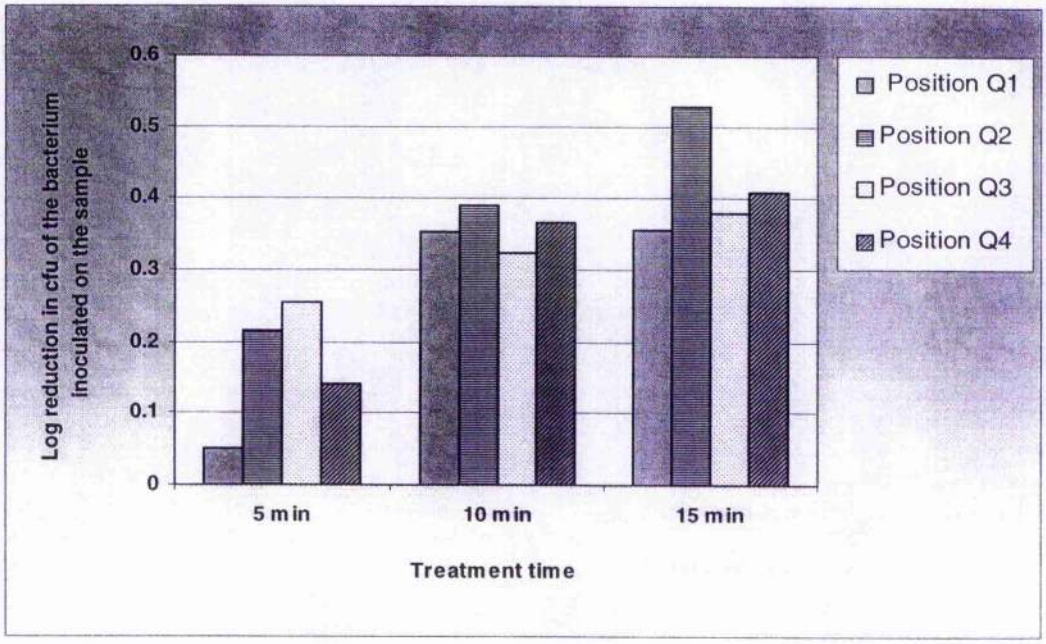


Figure 3-91. Reduction by ozone of the viability of *S. typhimurium* on chicken skin

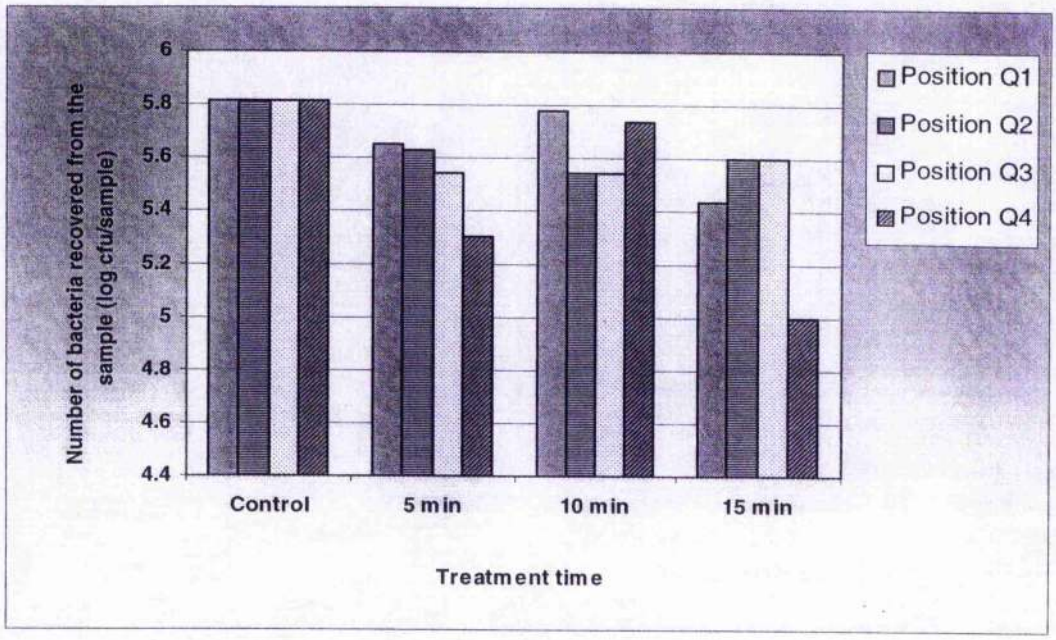


Figure 3-92. Killing of *S. aureus* on chicken skin by ozone

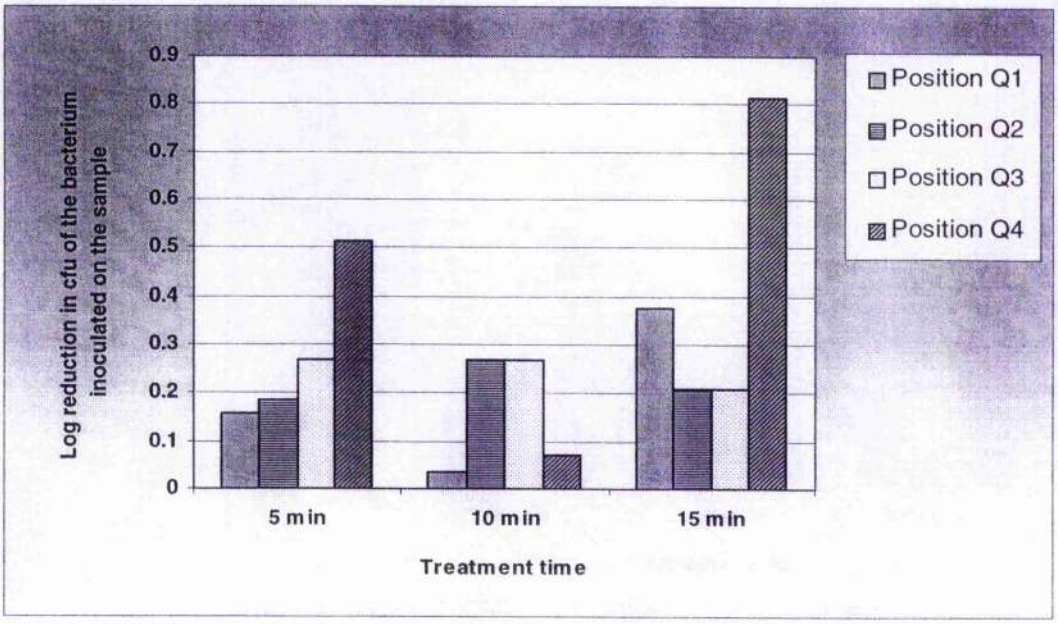
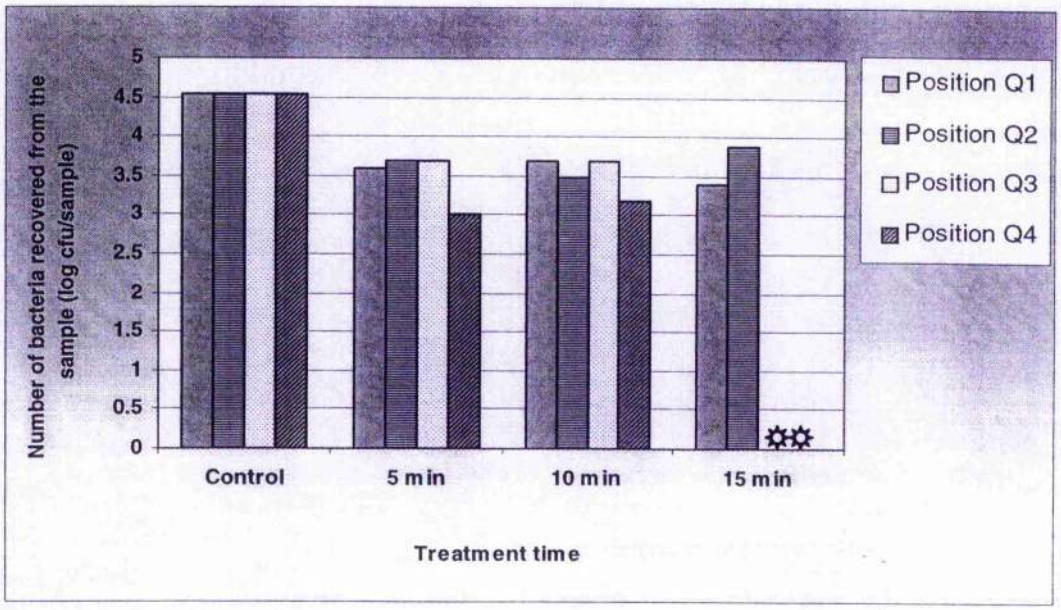
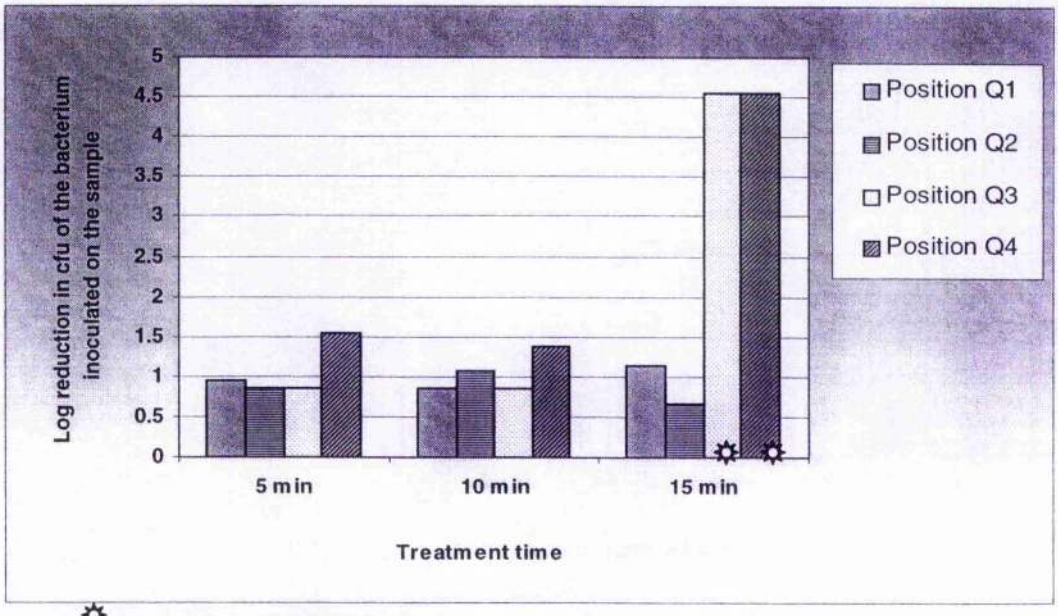


Figure 3-93. Reduction by ozone of the viability of *S. aureus* on chicken skin



☼ No colonies were found

Figure 3-94. Killing of *C. jejuni* on chicken skin by ozone



☼ No colonies were found

Figure 3-95. Reduction by ozone of the viability of *C. jejuni* on chicken skin

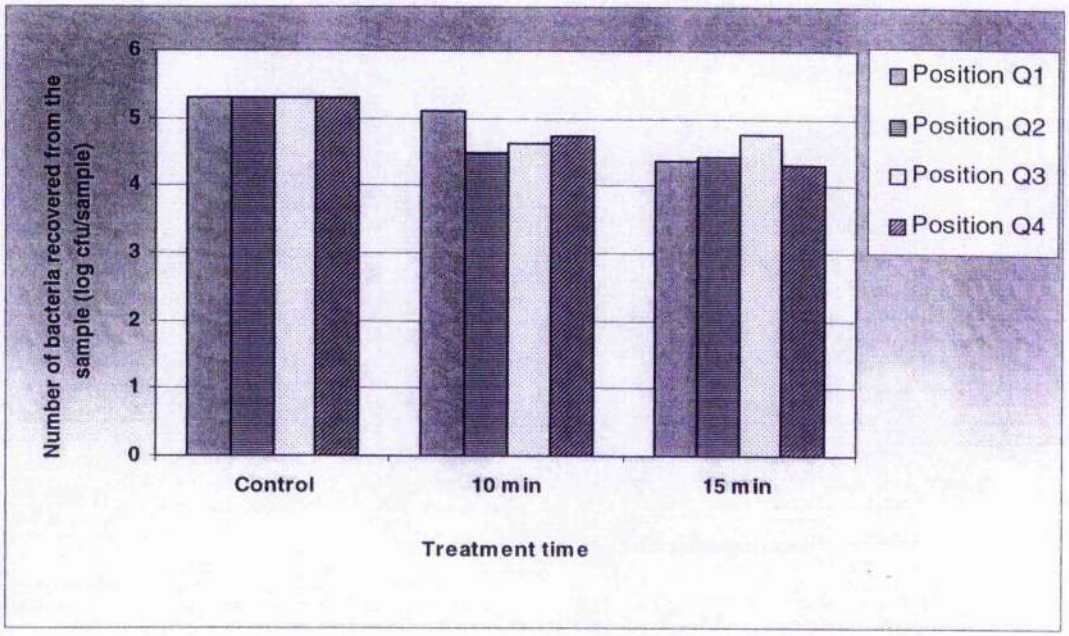


Figure 3-96. Killing of *L. monocytogenes* on chicken skin by ozone

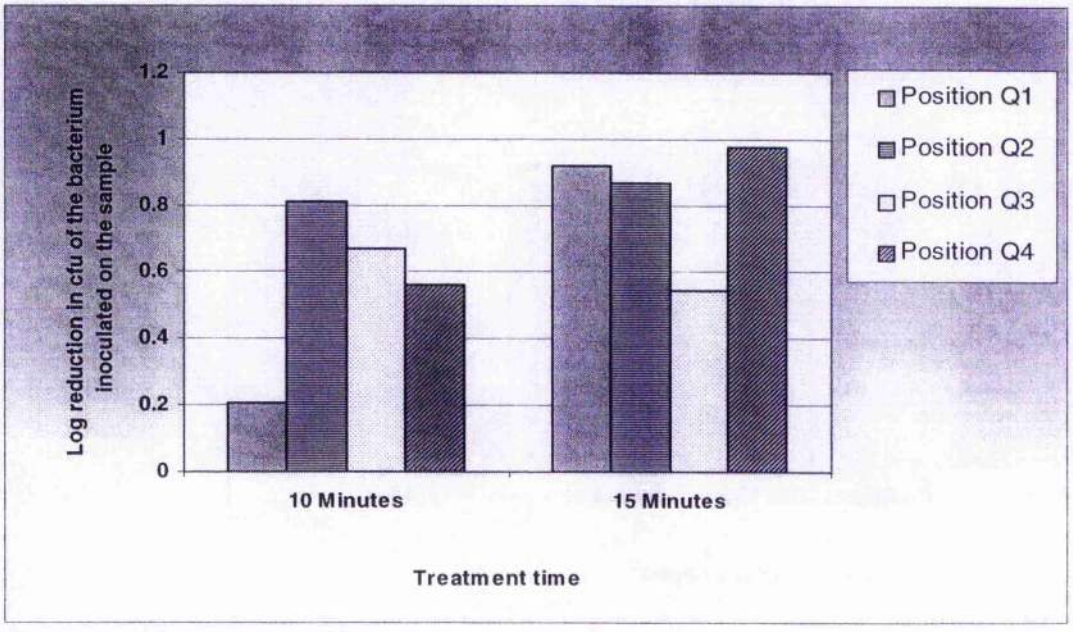


Figure 3-97. Reduction by ozone of the viability of *L. monocytogenes* on chicken skin

CHAPTER 4 DISCUSSION

Over the years, many different methods have been used to protect food against spoilage to increase their shelf life and to improve their safety. But there are limitations in the current methods of processing for microbial decontamination. Outbreaks of food borne illnesses have increased in the last decade and also vast amounts of food are lost annually due to microbial spoilage. Seafood is amongst the most perishable of foodstuffs and novel methods to improve the quality and extend the shelf life of these foods would be advantageous. More recent new techniques such as UV, laser, microwave, pulsed electric field (PEF), chemicals etc. have been investigated for decontamination of bacteria important in the food industry (see Introduction). In this study, the killing effect of UV, laser, microwave and ozone was investigated, alone and in combination, on some spoilage and pathogenic bacteria important in seafood and on *E. coli (lux)* as an indicator organism.

4.1 Killing effect of UV alone on bacteria

UV irradiation has been shown to be effective in killing many microbes and has been investigated in water, agar plates and different foodstuffs (85, 103, 176, 181, 216). **Table 4-1** shows UV doses typically required to inactivate different groups of microorganisms.

Table 4-1. D_{10} inactivation doses (i.e. dose required to reduce population viability by one order of magnitude) of UV (wavelength 253.7 nm)

Microbial group	UV dose (mW s/cm^2)
Bacteria (including spores)	0.4-30
Enteric viruses	5-30
Fungi	30-300
Protozoa	60-120
Algae	300-600

Data from (168)

In the present investigation, bacterial suspensions (1 ml volumes) were placed into multiwell petri dishes (1.7 cm internal diameter) and exposed to the UV light for different times and distances from the lamps. The depth of the exposed suspension was about 0.9 cm. *E. coli (lux)* was more sensitive to the effect of UV than *Pseudomonas fragi, Shewanella putrefaciens*, and *M. luteus*. More than a 6-log reduction in the viability of *E. coli* was caused by approx. 2.7 mW s/cm^2 of UV radiation after an exposure time of 5 sec, whereas the same energy density gave 2.55 and 2.92 log reductions, respectively, in the viability of *P. fragi* and *S. putrefaciens*. *M. luteus* proved to be the most resistant bacterium, with 11 mW s/cm^2 was required for about a 2-log reduction in viability. So, clearly the Gram-negative bacteria were more sensitive to UV than the Gram-positive bacterium used in this study.

These results can be compared with those of previous investigations: For example, UV radiation 2.7 mW s/cm^2 gave a 5-log reduction in the viable counts of *Vibrio anguillarum*, *Vibrio salmonicida* and *Yersinia ruckeri* in brackish water (about 1 cm depth in petri dish), whereas exposure to 122 mW s/cm^2 produced a 3 log reduction in the viability of some strains of viruses (111). In another study, 3-log reduction in the viability of *Campylobacter jejuni*, *Yersinia enterocolitica* and *E. coli* was observed when 1.8, 2.7 and 5 mW s/cm^2 of UV radiation, respectively, were used on bacterial suspensions (20). It was concluded that *C. jejuni* and *Y. enterocolitica* were more sensitive to UV than many of the pathogens associated with waterborne disease outbreaks. However, in each investigation, conditions of target sample e.g. volume, depth, concentration of sample etc. as well as sensitivity of different bacterial strains will play an important role in killing by UV radiation.

UV has also been used in killing bacteria on surfaces such as agar plates and on different foodstuffs. UV was effective in killing *Listeria monocytogenes* on agar plates by different powers, exposure times and distance from the plates (181). For example, at 30 cm (distance from lamps to organisms), 4 lamps generated 4.07 mW/cm^2 of energy and reduced the *L. monocytogenes* load from 636 (STDEV, ±281) cfu (control) per plate to 130 (±28), 8 (±5), and 0 cfu per plate at 1, 5 and 10 seconds, respectively. The authors concluded that the application of UV could be useful in the food manufacturing industry in reducing the microbial load on e.g. raw hotdogs, uncooked chicken and smoked

salmon. In another study, treatment of the surface of fish by 0.3 mW/ cm^2 of UV radiation for 16.6 min gave 2-3 logs reduction in the total viable count (85). The treatment made the shelf-life 7 days longer than that of conventional ice-packed untreated controls. However, the authors emphasised that UV was less effective on rough-surfaced fish than smooth-surfaced fish. So, they suggested that a combination of UV and chlorine could be used for reduction of surface counts on rough-surfaced fish to the same extent as that of UV alone on smooth-surfaced fish. A similar conclusion was published by Stermer *et al.* (180), when they exposed fresh meat to UV irradiation. The total count was reduced by 2 logs on smooth-surfaced beef plate meat by a radiation dose of 275μW/ cm^2 for 550 s. UV was less effective on rough-surface cuts of meat such as round steak because bacteria were partly shielded from the radiation. UV had no deleterious effects on the colour or general appearance of the meat. Their experimental results indicated that UV irradiation of meat carcasses could effectively increase the lag phase of bacterial multiplication until adequate cooling had occurred. In another study, *E. coli* was shown to be more resistant to UV treatment compared to *Salmonella senftenberg* on pork skin and pork muscle (216). Also, the killing effect of UV on *Salmonella typhimurium* was studied on agar plates and poultry skin (183). The data showed that UV was less effective on killing the bacterium on poultry skin than on agar medium. The viability of the bacterium was reduced by 99.9% on agar plates by 2 mW/cm^2 of UV radiation, whereas the value was 80.5% for the surface of poultry skin. The authors suggested that a combination of several techniques including UV radiation, in conjunction with good processing plant sanitation, might be effective in reducing bacterial loads in the poultry industry and would be an alternative to the use of gamma radiation.

In conclusion, many authors have indicated that the application of UV is potentially useful in the food manufacturing industry and have suggested that more research should be done in this area to determine the antimicrobial activity of UV light on different foodstuffs and the effectiveness of UV in extending the shelf-life of foodstuffs in cold storage. For example, mechanical flexing of the meat has been suggested to expose bacteria that may be embedded in the fibres (180, 181, 216). The present investigation has shown that UV alone at doses of 2.7 mW s/cm^2 can be highly effective in reducing

bacterial numbers in saline suspensions (by 2 - 6 logs i.e. by 99 - 99.9999% killing) and has a potential for use on foodstuffs with smooth surfaces.

It has been reported that, the killing effect of UV radiation was reduced when it was applied to foodstuffs compared to agar surfaces. This may have happened due to the fact that UV radiation does not penetrate most opaque materials and bacteria could have been partly shielded from the radiation. To have efficient killing on foodstuffs surfaces, the technique must be improved by exposing all angles of the food to the UV radiation. Using several lamps situated at different angles around the targets or using a treatment chamber with reflective walls could be helpful. Also, further investigation would be needed to ensure that such doses of UV irradiation would not affect the organoleptic qualities of the particular food and that treatment did not damage the food structure so that the shelf-life was decreased rather than increased, by favouring the growth of any surviving organisms. It may be that the dose of UV radiation could be reduced by combination of UV with other forms of treatment, as investigated in the present study.

4.2 Killing effect of microwave alone on bacteria

Although the main application of microwave radiation is for cooking and warming of food in a short time, it has been shown that the technique can reduce the population of pathogens and other microorganisms in foods and other media e.g. milk, shrimp, liquid culture and cooked poultry (70, 75, 146, 193, 199). The most important problem with microwave is the control of temperature in the exposed sample during treatment. The killing effect of microwave radiation has been investigated on many bacteria in foods and there has been much controversy over its killing mechanism.

The current investigation showed that microwave treatment was effective in the reduction of viable bacteria in saline suspensions. Although *E. coli (lux)* and *M. luteus* were slightly more resistant to the treatment than *S. putrefaciens* and *P. fragi*, it was unclear which bacteria were most sensitive to microwave treatment. It was noticeable that control of accretion of temperature with time of exposure was very difficult and the volume of the sample and the size, shape and place of the container in the microwave oven during treatment were also important. A bacterial suspension (50 ml) was placed in

200-ml conical flasks and the flasks was placed in the central cavity of a domestic microwave oven and exposed to microwave radiation without rotation for different times. Each experiment was repeated three times but the temperature of the suspensions at each repetition varied. For example, the temperature varied between 50 and 58°C after 15 sec of treatment and between 71 and 73°C after 20 sec. It was found that killing was related to temperature rather than to exposure time. When the temperature reached 71-73°C no viable organism of any of the 4 species were detected. In contrast, in two cases (**Tables 3-7 and 3-8**) when the suspensions were exposed to microwave for 21s but the temperatures were under 71°C, many colonies were detected.

Data suggested that, under the above experimental conditions, a temperature between 70-71°C was the critical point for killing bacteria by microwave energy. Microwave treatment is simple and rapid, but it is not easily controllable for food that may be different in shape, size, water content etc. As microwave energy involves heating, it is only suitable for food that is going to be heated. Heating and killing effects by microwave may be improved by making new generation of systems with several sources of different frequencies or in combination with other methods.

4.3 Killing effect of laser alone on bacteria

There are few reports of the investigation of laser light for applications in the food industry. In this project, the Nd:YAG laser was used for treatment of different bacteria in suspension. Data showed that the laser was effective in killing bacteria in a short time. Although *M. luteus* proved to be the most resistant bacterium, no significant difference was observed in the laser energy density required for killing this Gram-positive bacterium and the Gram-negative bacteria investigated. Survival of *S. putrefaciens*, which was the most sensitive bacterium, was below the limit of detection (50 cfu/ml. >6-log reduction) after exposure to approximately 550 J/cm^2, whereas the corresponding values for *P. fragi, E. coli (lux)* and *M. luteus* respectively were 690, 760 and 830 J/cm^2.

The killing effect of Nd:YAG laser and CO_2 laser was also investigated on different bacteria on agar plates. In the Nd:YAG laser, the energy densities for making the zone

of clearing equal to the laser beam area were about 1900, 2300, 2700 and 2900 J/cm^2 respectively for *S. putrefaciens, P. fragi, E. coli (lux)* and *M. luteus*. Thus, *M. luteus*, as a Gram-positive bacterium proved to be the most resistant bacterium and *S. putrefaciens* the most sensitive bacterium. The order of sensitivity of the bacteria to Nd: YAG laser on agar was same as that observed with suspensions.

With the CO_2 laser, energy densities delivered by continuous wave always gave clear areas greater by 0.5 to 0.7 cm^2 compared to those obtained with similar energy densities delivered by the pulsed mode, with different frequencies. Although *L. monocytogenes* is a Gram-positive bacterium, its sensitivity to this CO_2 laser treatment on agar plates was very similar to that of the Gram-negative bacterium *S. putrefaciens* rather than to that of *M. luteus*. This was confirmed by using continuous wave at different powers (20, 50, 100 and 500 W).

With the CO_2 laser, the energy densities required for making the zone of clearing equal to the laser beam area were much less than that observed for the Nd:YAG laser. For example, by using continuous wave at 20 W power output, approx. 14, 17.5 and 26 J/cm^2 respectively were needed to make the clear area equal to the laser beam area (0.35 cm^2) for *S. putrefaciens, L. monocytogenes* and *M. luteus*. This compared with 1900, 2300, 2700 and 2900 J/cm^2 for the Nd:YAG laser (pulse energy=20 J). To make the same clear area with the CO_2 laser with higher power outputs, 50, 100 and 500W, much smaller energy densities were sufficient. So, it was concluded that the same zone of clearing could be achieved by using higher power outputs and lower energy density. However, it is noticeable that using higher power on agar and similar surfaces is limited because a burning effect on the agar was observed, even before the clear areas were achieved equal to the beam area.

In comparison of the two laser types, the energy density needed for the Nd:YAG laser was approximately 300 times more than that needed by the CO_2 laser to produce the same clear area on the agar plates. This difference is believed to be partly due to the much higher absorption of radiation at 10.6 μm (wavelength of CO_2 laser) than at 1.06 μm (wavelength of Nd:YAG laser) by water in the bacterial cells and the surrounding medium (213). *S. putrefaciens* was the most sensitive bacterium and *M. luteus* was the most resistant bacterium to both lasers. Higher frequencies of the Nd:YAG laser resulted

in improved clearing effects and, with the CO_2 laser, continuous wave always showed better clearing compared to pulsed wave.

A preliminary study suggested that the laser radiation had no significant effect on the nutrient content and lipid oxidation of laser-treated ham (212). This and the above data indicate that laser light is an effective way of killing bacteria in liquids and on surfaces and could therefore possibly find some application in the food manufacturing industry, but more research is needed in this area. It has been shown that laser radiation is effective on killing bacteria and spores on different materials and it could be used for sterilization of surfaces e.g. surgical instruments, food packaging etc.

4.4 Killing effect of ozone on bacteria in agar plates and foodstuffs

It is well known that ozone is a powerful antimicrobial agent. Ozone could be suitable for application in food in the gaseous and aqueous states. Aqueous ozone has been investigated for decontamination of beef and beef brisket fat (60), poultry meat (37) cited by (98), and salmon (56) cited by (98). Gaseous ozone was tested for prevention of growth of surface contaminants of meat (69).

In the present study both types of ozone treatment were investigated for decontamination of different bacteria, *E. coli (lux), S. typhimurium, C. jejuni, L. monocytogenes and S. aureus,* on agar plates, chicken skin and smoked salmon. The gaseous ozone treatment involved the use of 2 chambers, namely the measurement and the treatment chambers. Four samples (inoculated-agar plates or food samples) were placed into the treatment chamber, in the four quarter positions, Q1 to Q4, (**Figure 2-2**) the samples were treated with ozone gas for 5, 10 and 15 min and colony counts were made before and immediately after treatments. With the aqueous treatment, 100 ml of bacterial suspension was treated with ozone gas bubbled through the suspension and colony counts were made before and immediately after treatment. Exposure times were 2, 5, 10 and 15 minutes. All the results consistently showed that ozone was effective against all strains of the bacteria tested during the experiments. The Gram-positive organisms were only slight more resistant to ozonation than the Gram-negative.

With gaseous ozone, the position of the samples in the treatment chamber was important. When seeded agar plates were exposed to ozone, the killing effect was not homogenous with a short treatment time (2 min) where, in all experiments, plates Q2 and Q4, which were positioned closer to the inlet ozone gas (**Figure 2-2 and 3-19**), showed more extensive clearing than plates Q1 and Q3, which were located at the far end of the chamber. This was again observed when food samples were treated by ozone. The results clearly indicated that, in the case of treating the sample with gaseous ozone over a short duration, the concentration of gas within the chamber might be uneven, resulting in uneven killing. With longer periods of treatment, this non-homogenous distribution of gas appeared to be less significant. Thus, the method of application of ozone must be improved to ensure even distribution and contact of the gas with the target microbial cells.

Ozone inactivates microorganisms less effectively when they are on food surfaces than in low ozone-demand liquid media. Inactivation of micro-flora on food by ozone depends greatly on the nature and composition of the food surface, the type of microbial contamination and the degree of attachment or association of the microorganisms with the food (101). Thus, in general, and in agreement with these previous findings, the present study showed that the killing of bacteria inoculated on the foodstuffs, namely chicken skin and smoked salmon, was less significant than killing on plates. Also, some discoloration was observed in the smoked salmon and chicken skin samples after their treatment by ozone for 15 min. Although this technique is beneficial in reduction of microbial loads, it could suggest that applying ozone at doses that are large enough for effective decontamination may change the organoleptic qualities of these products. It may be, therefore, that a combination of ozone treatment with other technique(s) should be used in order to reduce the dose of ozone. This combination probably will be more important in fatty products.

A reaction between ozone and lipids occurs at the carbon-carbon double bonds present in unsaturated fatty acids, producing different toxic products such as hydrogen peroxide, hydroxydroperoxides and aldehydes. These can cause rancidity in lipids and change the sensory evaluation. A combination of ozone and chlorine was studied by Gyurek *et al.* (72). They found that free chlorine was relatively ineffective against *Cryptosporidium*

parvum oocysts unless it was preceded by a small dose of ozone. Also, a combination of ozone and pulsed electric field (196) was used for inactivation of *Lactobacillus leichmannii, E. coli* and *L. monocytogenes* in 0.1% NaCl suspension. Treatment of *L. leichmannii* with PEF (20 kV/cm), after exposure to 0.75 and 1.0 μg/ml of ozone, gave a 7.1 and 7.2 log reduction in the viability of the organism, respectively. They reported that when *E. coli* and *L. monocytogenes* were treated with ozone and PEF, less pronounced synergistic bactericidal effects were observed. In another study, Ohshima *et al.* (142) reported a synergistic effect of the simultaneous application of ozone and PEF on *E. coli*. However, it was believed that ozone and PEF combination, as tested in that study, had an additive rather than a synergistic action (98). It is noticeable that chlorine, similar to ozone, is an oxidant and application of two oxidants on fatty products may have extra undesirable effects on organoleptic quality. Combination of ozone and PEF is only applicable in liquid products. Also, combination of ozone and UV radiation has been investigated (42) and a synergistic effect was found even when they were used in series. Similarly, a synergistic reduction in aerobic plate count bacteria was observed for ozone acting in concert with UV as compared with the sum of the effect of O_3 and UV acting in series (see section 1.7.5.1).

More investigation is necessary in this area to find out the best combination of techniques for the highest killing effect and the least adverse effects on the quality of the foods. Ozone can be applied easily on foodstuffs in two states, aqueous and gaseous. So, a wide range of combinations of ozone with other mechanical, physical, optical methods and other chemical agents are possible in order to reduce the adverse effects of the treatments.

In the current investigation, among the tested bacteria, *C. jejuni* was possibly the most sensitive bacterium to ozone treatment on the chicken samples, although these experiment need to be repeated. It is well known that the bacterium is microaerophilic, requiring reduced oxygen and increased CO_2 for optimal growth and it could explain why the bacterium is sensitive to ozone. *S. aureus* and *L. monocytogenes* were the most resistant bacteria to ozone treatment. This might be related to the outer layers of the bacteria. Previously, it has been shown that Gram-positive bacteria are more resistant to ozone than other strains (108). In another study, exposure of bacteria to ozone at 2.5 ppm

for 40s caused 5-6 log reduction in viable count and it was reported that *L. monocytogenes* was the most resistant bacterium to ozone treatment followed by *Leuconostoc mesenteroides, Pseudomonas fluorescens* and *E. coli* O157:H7 (101).

Ozone could be widely used in the food processing industry as a powerful disinfectant. It could be used to remove pathogens or to reduce the spoilage bacterial population on different foodstuffs. Although ozone may have some adverse effects on fatty products, many foodstuffs such as fruits, vegetables, dried foods, summer crops etc. could be disinfected by ozone. Ozonation does not need any heating procedure and so a wide range of liquid and solid foodstuffs could be treated. As suggested, a combination of ozone with other treatments could be even more effective on killing bacteria and so reduce the doses required for the same effect by each treatment alone. This may reduce the adverse effects of ozone on fatty foods and other foodstuffs, which may be sensitive to ozone or to other treatments. Also, ozone could be widely applicable for sterilization of food packaging material, equipments, surfaces etc. in the food industries. More investigations are required to find out the effects of ozone on different foodstuffs and particularly food ingredients.

4.5 Killing effect of combination of UV and laser on bacteria

Combined treatments were investigated to determine if any synergistic effect could be obtained between methods that killed bacteria in different ways. This would allow the application of least energy for each treatment to achieve microbial decontamination. Based on data obtained, no major synergistic effect between UV and laser treatment was observed with the Gram-negative bacteria but, with the Gram-positive bacterium *M. luteus,* better killing was apparent with combination of these two treatments, in comparison to the sum of the killing of each treatment alone. For example 11 sec of Nd:YAG laser radiation followed by 15 sec of UV treatment gave a 3.58 log reduction in the viability of *M. luteus*, which was 1.2 logs more than the sum of the log reductions by each treatment alone. With *E. coli*, combination of the two treatments, 10 sec laser followed by 5 sec UV, produced about 0.5 log greater reduction in viability compared to the sum of the two treatment alone. Also, with *E. coli* the order laser followed by UV

gave better killing than the order UV followed by laser. Increasing energies increased the synergistic effect.

This combination could be useful for inactivation of Gram-positive bacteria, which are more resistant to each treatment alone. As described before, UV and laser kill in different ways, by acting on different cell targets, and combination of the both treatments could be more effective especially on thick-walled Gram-positive bacteria. An increase of 1-log reduction in viability by combination of UV and laser means 90% more killing with the same energy densities used. It would be interesting to confirm these results by using wider a range of organisms.

4.6 Combination of UV, laser and microwave

Combination of three treatments, each with low energy and relatively low effects, was done on *E. coli (lux)* and *P. fragi* in order to determine whether any synergistic killing effect occurred. The three treatments were applied in different orders. Sequential treatment by UV, microwave and Nd:YAG laser gave much greater results than the sum of the three treatments alone. More than a 5.82 log reduction in viability of *E. coli* was apparent when treatment was in the order: laser, microwave and UV, whereas the sum of the log reduction in viability by the three treatments alone was only 2.66. Thus, combination of the three treatments gave approximately 1000-fold greater reduction in the viability compared to the sum of the three treatments. For *P. fragi,* about 0.91 log greater killing was found compared to the sum of the log reduction in viability of the three treatments alone. The best sequential order for this bacterium was microwave, UV then laser. So, under the experimental conditions, it was concluded that the synergistic effect was more apparent on *E. coli (lux)* than on *P. fragi.* The order of the treatments was important in the overall reduction in viable count. It seems that the best order for each bacterium may vary. As described in Section 3.4, the experiment was repeated 3 times and the killing effect of microwave was variable in the 3 experiments. The reason for the differences between treatments and organisms could possibly have been due to the difference of temperatures achieved by microwave radiation and the difficulty in standardizing the exposure of the samples to microwave radiation. It was unclear whether

the differences between the killing effects of the different orders of the treatment was due to the sensitivity of the strains to the orders of treatment or due to the variable killing by the microwave. For this reason, microwave treatment was placed by conventional heating in a water bath for subsequent experiments.

4.7 Combination of UV, laser and conventional heating

Again, a consistent synergistic effect was apparent in killing bacteria when a combination of the three treatments was used and compared to the reduction in viable counts caused by the individual treatments. As in the previous experiment, the more effective order for killing was laser then heating then UV and the least effective order was heating then UV then laser. The experiment was repeated 3 times and although the results statistically were not significant the sequential order treatment L+H+UV produced a 1.83 log reduction in viable count of *E. coli*, whereas the value for the least effective order H+UV+L was 1.15. The other orders of treatment gave between 1.4 to 1.68 log reductions in viability. Under standard conditions, the priority of the order L+H+UV over the order H+UV+L was seen consistently through the different experiments for *E. coli*. Statistics showed that the difference was significant ($P<0.01$). Differences between the best and worst orders of treatment were increased when more severe treatment conditions were used. This difference varied between 0.7 log reductions ($P<0.01$) in viability with less severe treatment condition (8 sec laser radiation, 5 sec UV radiation and 5 min conventional heating at 50°C) and 1.3 log reduction ($P<0.001$) for the highest used paramcters (9 sec laser radiation, 10 sec UV radiation and 5 min conventional heating at 55°C). As can be seen, the difference between the best order and worst order when more severe treatment conditions were used was more highly significant.

One possible explanation for the apparent synergistic effect of the combined treatment was that a higher temperature could have been reached by following laser treatment with heating. To investigate this further, the bacterial suspensions were cooled to a standard temperature (25°C) between treatments. When this was done, an interesting observation was made which suggested different killing mechanisms by heat and laser. When UV was

used after laser, the killing effect was almost double that seen after heating, whereas the killing effect of laser and heat alone were similar.

The combination of UV, laser and conventional heating was used also for treatment of *S. putrefaciens, P. fragi, M. luteus and L. monocytogenes*. Differences between the order L+H+UV and H+UV+L for these bacteria were 0.72 ($P<0.05$), 0.33 ($P<0.001$), 0.54 ($P<0.001$) and 0.20 ($P<0.01$) log reductions, respectively. It was found that the best and worst order might be slightly different for each bacterium, although different treatment parameters were used for each bacterium. More investigation is needed to find out the killing effect of different orders on each organism. The reasons for the differences between strains also need to be investigated further and with a wider range of organisms. Different sensitivities of each strain to heating or UV is one possible reason for these differences. More investigation, comparing sub-strains of each bacterium resistant and sensitive to heating or UV, heat resistant mutants, spores or vegetative cells would be of interest to find out whether microwaves or lasers acting the same way as conventional heating, by investigating whether these organisms are equally resistant to killing by different combinations. Some organisms are known to be highly resistant to UV and gamma radiation (e.g. *Deinococcus radiodurans*). Also, different organisms could be investigated further to find out if they are resistant to laser radiation. Combinations of different methods may be useful for inactivation of resistant or unusual organisms, which may cause specific problems in different areas.

Although laser, UV, microwave or heating alone with high energies potentially will kill bacteria, the possible advantage of combined treatments in applications on food is a reduction of contact times and energies. Results showed that by using the least energy in a suitable combination of treatments, greater killing of bacteria was obtained. So, by using combined treatments, removing pathogenic bacteria, or reducing the population of bacterial flora on a food could be achieved and produce hygienic food with a longer shelf life; hopefully without changing the taste, flavour of the food or losing essential labile vitamins etc. It is suggested that such combination of treatments might be useful in food processing but they could also have application in non-food areas e.g. surgical instruments, bacterial pollution areas, operating rooms, where antibacterial or disinfectant- resistant strains of microbes may be presented.

4.8 Killing effect of combined treatments on bacteria inoculated on smoked salmon

As discussed above, sequential combined treatment of laser, heat then UV had the best killing effect on *E. coli* in saline suspension. This order also gave a small (0.2 log) but consistently greater reduction in the viability of *L. monocytogenes* in suspension compared to the order H+UV+L. To determine the killing effect of separate and combined treatments on actual samples, smoked salmon was inoculated with *E. coli (lux)* and *L. monocytogenes*. The samples were then exposed to the UV light, laser radiation and conventional heating, alone and in combination. The killing effect of the two orders was compared. The sum of the killing effect of the three treatments on *L. monocytogenes* was 0.78 log reduction in viability, whereas the value for the order L+H+UV was 0.93 and for the order H+UV+L was 0.61 log reduction in viability. Although the value for the order L+H+UV was greater, the standard deviation was high (**Table 3-58**) and the results may not be statistically significant. Similar results were obtained when *E. coli* was exposed to these treatments. The differences between the best and worst order was even smaller (**Table 3-60**). Killing effects of 30, 60 and 90 ms of laser treatment on *L. monocytogenes* were almost same. Also, similar results were monitored for 8, 10 and 15 sec of UV treatment. One possible reason is that some bacteria may have been hidden under the rough surface of salmon flesh and were not exposed to the electromagnetic treatments so, only exposed bacteria had been killed. This affected the combination of the three treatments and may suggest that using this kind of combination treatment will be more useful on smooth surfaces or the technique need to be improved. Exposure of samples to UV light or laser radiation at different angles, possible mechanical flexing of the samples to increase exposure or as suggested in section 4.1, by designing treatment chambers with reflective walls may be useful.

4.9 Bioluminescence and viable count assay

A bioluminescent bacterial strain, *E. coli (lux)*, was used in some experiments as an indicator organism to measuring the efficiency of the different treatments and their combinations. Bioluminescence has been used as a real-time indicator of bacterial

viability in various systems. For example, Forde *et al.* (52) used bioluminescence for monitoring of intercellular survival of *Bordetella bronchiseptica* in murine phagocytes. In this study it was hoped that the bioluminescence assay could replace traditional viable counting which is expensive and time-consuming.

Initially, the correlation between bioluminescence output and *E. coli* viable cell numbers was determined with untreated suspensions. There was an almost linear relationship between light output and viable cell number between 10^9-10^7 cfu/ml (**Table 3-43 and Figure 4-1**). Below 10^7 cfu/ml, the light output was low but even 10^5 cfu/ml gave a light output above background. Below 10^5 cfu/ml the luminometer could not measure accurately light output in the suspension. So, the light output method was only good for measuring high numbers of bacteria with this particular *E. coli* DH5∝ strain. This strain contains plasmid PT7-3 containing the lux ABCDE genes from the naturally bioluminescent organism *Photorhabdus luminescens*. The genes are under the control of a phage T7 promotor but, even in the absence of the T7 polymerase the *E. coli* construct was bioluminescent due to the native lux promoter on the 7 kb *EcoRI* fragment in PT7-3. From the work of Szittner and Meighen (188) it is likely that the strain would be 10-fold brighter if a plasmid expressing T7 RNA polymerase were included in this bacterium. This would enhance the sensitivity of the detection system by 10-fold. It is possible that other, more highly bioluminescent constructs could be made, to further enhance the sensitivity of the system and allow the killing effect to be assessed more accurately.

With the present *E. coli* construct, and starting at 10^9 cfu/ml, only up to 2 log reduction in the viable counts could be determined accurately and down to a 4-log reduction could be detected. Nevertheless, the system did provide some useful information. Bacterial suspensions were exposed to different treatments and light output and colony counts were measured. Some interesting differences between treatments were apparent, which could indicate that treatments act in different ways. The different curves indicate very different effects of the treatments on light output and viability. For example, UV reduced the viability of the bacterium by about 8 logs, but the light output immediately after treatment was not significantly affected by UV treatment (**Figure 4-1**). UV is known to damage DNA and prevent bacterial replication, rather than destroy the integrity of the cell or denature proteins, which would include the light producing

integrity of the cell or denature proteins, which would include the light producing enzymes. Thus, use of bioluminescent bacteria does not provide a real-time method of assessment of killing by UV alone. In contrast, with laser treatment, the light output reduced dramatically without greatly reducing the subsequent viable count. A similar result was apparent for treatment with conventional heating. The results could suggest that the treatment with laser or conventional heating could destroy the light producing system before they greatly affected the cell integrity and viability.

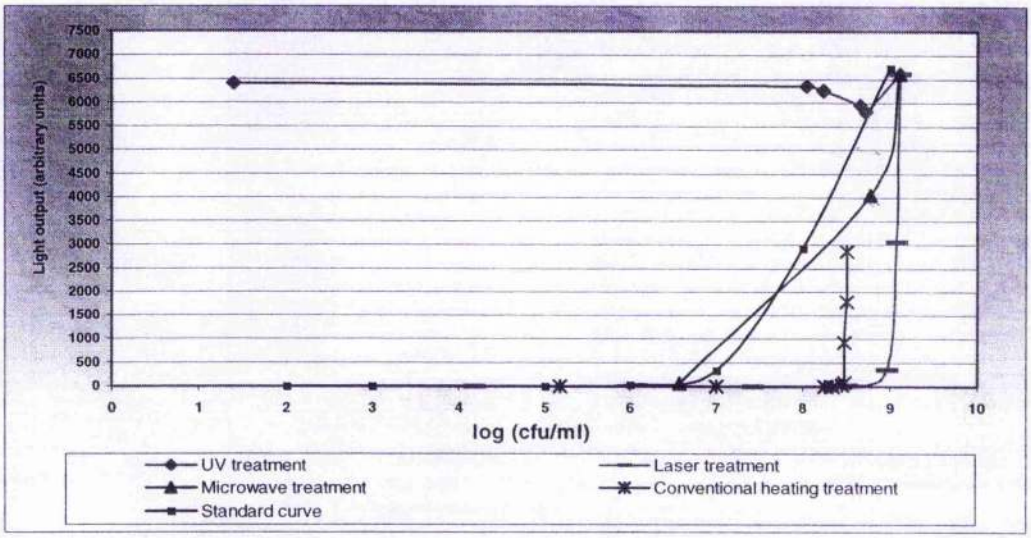


Figure 4-1. Comparison between viable counts and bioluminescence for *E. coli (lux)* after different treatments

Microwave treatment showed a rather similar pattern to the untreated cells, in terms of the relationship between light output and viable cell numbers, although only two microwave energy levels (10 and 15 sec treatment time) were investigated. When the killing curves for conventional heating and microwave were compared, they were very different which could indicate that they affect cells in different ways. As explained before, this could be due to an athermal effect by the microwaves in addition to its

thermal effect, a suggestion which has already been reported (46, 99, 184). These observations highlight the limitation of the use of the bioluminescence technique as a real-time monitor of bacterial viable cell numbers. Various factors such as temperature, growth rate, growth phase will effect the metabolic activity and therefore the light output from bioluminescent bacteria but, if standard conditions are used, perhaps with a more highly bioluminescent organism, it is possible that the method could be useful to study decontamination processes. Some treatment such as UV may not be suitable but, with others that damage cellular integrity, the bioluminescence method could provide rapid information on the processing method.

An attempt was made to detect low numbers of bioluminescent bacteria on lawned plates using a CCD camera. A suspension of the bacterium was made and different dilutions from 10^6-10^3 cfu/ml were prepared. An aliquot (20µl) from each dilution was placed on the agar as a drop. The plate was left to dry and then examined under the camera but it was not possible to detect any bioluminescence from the agar. Similar results were obtained when drops of each dilution were placed in a sterile petri dish with the camera set for highest sensitivity. However, a noisy background appeared and no drops could be seen. Again, it is possible that such techniques could be used for a very highly bioluminescent bacterium and with a more sensitive camera. Decontamination of solid surfaces e.g. of foodstuffs, could then be viewed in real-time.

4.10 Investigation of the killing mechanism

The killing mechanism of the different treatments separately and combined was studied in several ways. The effect of released cell constituents by one method of treatment on protection of bacteria against subsequent treatments was studied. Although there was some variability in counts of surviving bacteria, the results showed that the cell constituents released by the microwave treatment could protect bacteria against subsequent treatments by UV and laser radiation. In all cases, the reduction in viability was less in the samples where the bacteria were suspended in supernate from microwave-treated cells rather in supernate from untreated cells. This could possibly be explained by the fact that microwave heating would release DNA and protein which are known to absorb UV light (and, possible, laser light) and thereby reduce its action of the bacterium

itself. Other treatments i.e. conventional heating, laser and ozone, also cause release of cell constituents (see section 3.9.2) and could have a similar protective effect. Thus, it is suggested that, with combination treatments, killing by one treatment might be affected by cells constituents released from previous treatment(s). It may be that treatment by a method mild enough to create sub-lethal damage on the cells, without release of cell constituents, may be the most effective treatment at the start of the series treatments. Data with *E. coli* consistently showed that the order, L+H+UV, gave better killing than the order H+UV+L and these mild laser treatment would cause little release of cell contents. It is likely that laser radiation or a mild conventional heating with a mild energy, sensitised the live bacteria to be killed by subsequent treatment (s) or laser treatment had an intra-cellular effect.

Explanation of the effect of combination methods in different foodstuffs will be more complicated. It well known that the composition of the surrounding medium affects sensitivity of bacteria to heat or cooling. For example, foodstuffs with high levels of fat can protect bacteria against heat or cold shock more than other foods. Other food compositions such as carbohydrates, vitamins etc. also may change the effect of the treatments on bacteria. More investigation is needed to determine the effects of combination methods in different foodstuffs.

In another part of the investigation, the effect of different treatments on release of DNA, RNA and protein from bacterial suspensions was investigated. Results showed that, with all treatments, the greater the killing effect, the greater the released material (i.e. nucleic acids and protein). Release of nucleic acids and protein by different treatments varied and it may suggest different types of damage to the cells and different killing mechanisms by each treatment. Killing by UV did not release a significant amount of nucleic acid and protein. Measurement of OD at 260 and 280 nm after killing by conventional heating and microwave were completely different. For example, with similar killing by both methods, the observed OD of the supernate at 260 nm was almost double for suspension treated with conventional heating compared with that treated with microwave. The value for the OD at 280 nm was 1.5 times more. Again, it may suggest different killing mechanism of the treatments. A greater release was caused by

conventional heating than laser for the same reduction in viable count. All of these differences could show the different action on cells by the different treatments.

The effect of different cooling methods after microwave treatment on the effectiveness of subsequent treatments was investigated. Cooling at room temperature, by ice and by a mixture of acetone and dry ice were chosen to apply to *E. coli (lux)* and *M. luteus*. Controls without microwave treatment were included. Data showed that a quick freezing (by a mixture of acetone and dry ice) of the bacterial suspension after microwave treatment or perhaps after other heat methods not only could have a killing effect on the bacteria but also may increase their susceptibility to the killing effect of subsequent treatment(s). For example, freezing alone after microwave treatment gave 0.56 log reduction in the viability of *E. coli* and cooling by air did not give any reduction in the viability, whereas, treatment by laser alone after microwave treatment and cooling by air gave a 0.95 log reduction in the viability and the value after freezing was 1.52 log (**Table 3-52**). So, the total log reduction in the viability of *E. coli* by air-cooling and laser was 0.95 log, whereas the value for freezing and laser was 2.08 log. The combination of warming + cooling + a treatment (in this experiment laser), was more effective on *E. coli* as a Gram-negative than *M. luteus* as a Gram-positive bacterium. This method could be part of a decontamination procedure in the food processing industry but needs more investigation.

Sensitivity of the bacteria to lysis by SDS after different treatments was investigated as an alternative way to investigate cell envelope damage. This method had been used by Woo *et al.* (219) to investigate damage to the cell wall structure in bacterial cells by microwave radiation. *E. coli* cells treated with laser, microwave and conventional heating were much more sensitive to lysis by 0.1% SDS than control cells and more sensitive than the cell treated with ozone and UV. The OD_{600} in the suspension caused by SDS decreased from 0.09 to 0.01 after heating, microwave and laser treatment, whereas the value after ozone treatment was reduced from 0.11 to 0.08 and no significant reduction was observed after treatment with UV. The result clearly showed that laser, microwave or conventional heating have some effect on the cell envelope and sensitise the bacteria to be lysed by SDS, but these effects are probably lower for ozone and minimal for UV, which is well known to act primarily on DNA

The effect of various treatments on the morphology of *E. coli* was investigated by scanning and transmission electron microscopy. The bacterium was exposed, separately and in combination, to different treatments with low energies. Little damage observed by SEM and this was limited to observation of some misshapen or curved cells after treatment by laser, microwave and conventional heating. Some cell surfaces were wrinkled and rough when they were compared with untreated cells. Almost similar changes, as above, were seen after combined treatments even though considerable killing (more than 4-5 log reduction in viability) occurred. No damage was observed in the cells treated with UV. In a similar investigation, suspension of *E. coli* was exposed to Nd: YAG laser until the temperature of the suspension was raised to different degrees (40, 50, 60 and 70°C) and then the cells were examined by SEM (204). No significant damage were observed in the cell surface after low exposure times, i.e. raising the temperatures to 50°C but, when the suspension was exposed to laser light raising the temperature to 70°C, more visible damage such as blebbing was observed.

Findings in TEM were limited to observation of some vacuoles or less dense areas in the cytoplasm as a result of the laser and conventional heating treatments. No significant abnormalities were observed in the cells treated with UV. Almost similar findings were observed in cells treated by combined treatments, although up to 5.6 log reduction in viability (>99.999% killing) occurred in the bacterial suspension. All results indicated that no gross ultrastructural changes to the internal structures of the cell or rupture in the cell-envelope of *E. coli* occurred after different treatments.

4.11 Killing of bacteria with SDS

SDS is an anionic detergent and can be used to lyse bacterial cells. The susceptibility of damaged cells to lysis by a low concentration of SDS (0.1%), described above, to determine the degree of damage to cell integrity suggested the use of SDS as part of a combined treatment. SDS 0.5% was able to reduce the viability of a suspension of *E. coli* by 0.74 logs at 25°C and 1.67 logs at 50°C. An interesting result was obtained when *L. monocytogenes* was exposed to different concentrations of SDS. Data showed that the bacterium was much more sensitive to this treatment. SDS 0.01% gave about 0.22 log

reduction in viability after incubation of the bacterium for 15 min and more than 4 log reduction after incubation for 30 min at room temperature. A synergistic effect was apparent when *L. monocytogenes* was subjected to different treatments such as laser, UV, conventional heating and then exposed to SDS 0.01%. The best result was achieved by combination of 10 sec laser and SDS, where more than a 2-log reduction in viability was monitored for incubation of the cells in the SDS 0.01% for 15 min. Combination of heat or UV with SDS also, gave a significant reduction in viability of the bacterium. After treatment of the bacterial suspension with conventional heating or UV, incubation of the cells in SDS for 15 min gave a 1.67 and 1.46 log reduction in the viability, respectively. The data suggest that SDS potentially could be used alone or in combination with UV or heating as effective and cheap methods for inactivation of *L. monocytogenes* in the food industry. It is well known that there is a high incidence of *L. monocytogenes* in smoked salmon factories and contamination of cold-smoked salmon is due to contamination during processing rather than to contamination from the raw fish (201). So, control of the bacterium in the processing area (equipment, surfaces etc.) could be useful to reduce the numbers of the bacterium in the final products. Although the application of SDS on smoked salmon or other foodstuffs may be undesirable or prohibited, a low concentration of SDS in hot water or in combination with UV or laser could be used for decontamination of the bacterium or perhaps other bacteria in the processing area. Even if SDS can not be used in or near foodstuffs, it is possible that other detergents, anionic, cationic, non-ionic or zwitterionic, could be used in its place and have a synergistic role in decontamination. However, application of different concentrations of SDS or related substances in combination with laser, UV or heat may be useful for bacterial decontamination of other materials and surfaces.

4.12 Investigation of seafood

L. monocytogenes has frequently been isolated from cold smoked salmon. **Table 4-2** shows examples of prevalence of the bacterium in different countries.

In the present study, different seafoods, mostly smoked salmon, were investigated for *Listeria* species and also for aerobic viable total count at 18, 30 and 37°C to determine

the bacteria load. In total, 24 samples were investigated, 17 samples were chilled smoked salmon. Two smoked salmon samples (11.8%) were found to contain *L. monocytogenes*. These were isolated on the Listeria selective medium (Oxford agar) by using an enrichment step with Listeria enrichment broth. The isolated bacteria were confirmed as *L. monocytogenes* by the API® Listeria system. No other *Listeria* spp. were isolated from the samples. 19 other unidentified bacteria, 15 Gram-positive and 4 Gram-negative, were grown on Oxford agar and Listeria monocytogenes blood agar. The results indicated that, smoked salmon may contain *L. monocytogenes* and, as discussed before, this may be due to initial or post-processing contamination (201). Although no cases of listeriosis have so far been directly linked to smoked-salmon, the presence of *L. monocytogenes* in this product is a potential problem for consumers, especially for the elderly, children and pregnant females. As discussed above, using a suitable detergent alone or in combination with other techniques, could remove the bacterium from the contaminated foodstuffs or from the processing environment.

4-1. Prevalence of *L. monocytogenes* in seafood samples

Sample	Prevalence (%)	Country	Reference
Salmon	25	Canada	(48)
Smoked seafood	16.7	Canada	(44)
Cold-smoked salmon	75	New Zealand	(86)
Cold-smoked salmon	9	Norway	(155)

The highest population of micro flora was found at 30°C. The mean of total count at this temperature was 1 x 10^6 cfu/g of samples, whereas the values at 18 and 37°C, respectively, were 5.1 x 10^5 and 3.2 x 10^5 cfu/g of samples. This showed that the optimal temperature for growing the surface micro flora is 30°C. In two samples the bacterial level was more than the average. These samples were nearing their last sell-by date when they purchased and examined and the level of bacteria was probably increasing.

4.13 General conclusions

Data showed that all treatments, UV, laser, ozone and heating were effective in killing bacteria and could be used in the food industries. A synergistic effect on killing was apparent when combinations of the treatments were used. Although all investigations on combined treatments were done sequentially, it may be that the killing effect of treatments could be improved if they were applied in concert. By combination of the treatments, less energy by each treatment can be used in order to save essential food ingredients and prevent adverse effects on the foodstuffs. The combination of these more recent techniques could be used to reduce the spoilage bacteria or remove pathogens during processing or packaging and before storage and, therefore, play an important role in making food safe and providing a longer shelf-life. The combined techniques also could be a suitable way to destroy unwanted resistant bacteria, spores or other organisms in the food industry. Although, in this thesis, the focus of interest was on the problems of spoilage and prevention in relation to seafood, the results could be extended to the other problems or surfaces e.g. packaging material, surgical instruments, infected areas such as hospitals, different liquids, plastics, metals etc. Also, with using a suitable combination, the techniques could be used for control of pathogens in animal husbandry.

Release of nucleic acids and protein by different treatments was varied and suggested that damage to cells occurred in different ways. There was a significant difference in release of cell constituents between conventional heating and microwave treatment. Also, a greater release was observed for conventional heating compared to laser treatment. It was also found that laser, microwave or conventional heating sensitise the bacteria to be lysed by SDS, but these effects were lower for ozone and minimal for UV treatment.

It was shown that *L. monocytogenes*, a potential pathogen in the seafood industry, was highly sensitive to SDS and also there was a synergistic effect between SDS and other treatments on killing of this bacterium. If SDS cannot be used in or near foodstuffs, it is possible that other detergents could be used in place of SDS and have a synergistic role in decontamination of seafood factories or other materials and surfaces.

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